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ASPERGILLUS NIGER GROUP

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OXALIC-ACID PRODUCTION OF SPECIES OF ASPERGILLUS

The recent discovery by the writers of an oxalic-acid-forming species of Penicillium [1] led from a review of the subject of oxalic-acid production by molds to a study of the black forms of *Aspergillus* spp. Wehmer, in 1891,[2] showed that *A. niger* is a very active oxalic-acid-producing fungus. The question whether this ability to produce oxalic acid is possessed in equal degree by all strains of *A. niger* has not been heretofore discussed. Culture 111, received from Amsterdam, Netherlands,[3] as *A. niger*, was selected for comparison with *Penicillium oxalicum*, because this strain was supposedly obtained originally from Wehmer.

Aspergillus niger has been the subject of a large number of biochemical researches. Back of this selection lies its apparent ease of specific identification, together with, as a corollary, the assumption that the study may be repeated elsewhere by isolating a black species of Aspergillus. Antithetic to this point of view there occurs in the literature a series of specific names and descriptions of black or dark-brown species, most of which rest upon minor morphological characters plus the assumption that occurrence upon hosts or substrata of widely different nature is evidence of specific difference.

Wehmer, in 1901,[4] cited 18 such names; at least 25 may now be found in the literature. Comparative culture may ultimately show how many of these may be separated by characters definite enough to be used in descriptive work.

The possible bearing of the comparative study of oxalic-acid production upon the problem of relationship among this lot of strains or

[1] Currie, J. N., and Thom, Charles. An oxalic acid producing Penicillium. *In* Jour. Biol. Chem., v. 22, no. 2, p. 287–293, 1 fig. 1915.

[2] Wehmer, Carl. Entstehung und physiologische Bedeutung der Oxalsäure in Stoffwechsel einiger Pilze. *In* Bot. Ztg., Jahrg. 49, p. 233ff. 1891.

[3] By courtesy of Dr. Johanna Westerdijk.

[4] Wehmer, Carl. Die Pilzgattung Aspergillus in morphologischer, physiologischer und systematischer Beziehung unter besonderer Berücksichtigung der mitteleuropaeischen species. V. Systematik. C. Schwarzbraune Arten. *In* Mém. Soc. Phys. et Hist. Nat. Genève, t, 33, pt. 2, no. 4, p. 103–111. 1901.

species led to the selection of 20 cultures for a comparative test. Two forms which could be readily distinguished from the typical black group were included: 4030.4, a strain of *A. ochraceus*, and 3522.30, possibly *A. violaceo-fuscus* of Gasperini. Two other forms, 2580 and 4030.1, have differences discoverable readily with the microscope; but most of the series shade into each other morphologically. The history of these forms, so far as known, follows: 111, *A. niger*, received in September, 1909, by courtesy of Dr. Johanna Westerdijk, Amsterdam, Netherlands; 3534–a, *A. niger*, var. *altipes*, 3534–b, *A. cinnamomeus*, 3534–c, *A. fuscus*, the three forms described by Schiemann [1] as mutants from a strain of *A. niger* obtained from Amsterdam and probably identical with No. 111; 142, marked *A. ficuum*, P. Hennings, received from Amsterdam; 2396, from Missouri, by Prof. G. M. Reed; 2580, isolated from interior of red pepper (capsicum) from Barcelona, Spain; 2469.4, from Delaware, marked *Sterigmatocystis violacea*, by Prof. M. T. Cook; 2657, from soil, England, by Miss E. Dale; 2774, from ulcerated human ear, by Dr. A. B. Stout; 2766, from fermenting mash, consisting of oak galls from China; 3522.30, possibly *A. violaceo-fuscus* of Gasperini from soil, Porto Rico, by Dr. J. R. Johnston; 3528.7, from Pittsburgh, the Mellon Institute, by Mr. F. A. McDermott; 4049, from sardine paste, Bureau of Chemistry; 3547.254–b; from Kansas soil; 4050, from Chinese galls, sent by Eastman Kodak Co., 4020.33, from soil, Texas; 4030.1, *A. carbonarius*(?), 4030.4, *A. ochraceus*, 4030.5, from Dr. A. F. Blakeslee, Storrs, Conn.

Table I gives the chemical results with the strains arranged approximately in the order of their relative activity in oxalic-acid production. Two determinations are given: The direct titration of free acid expressed in cubic centimeters of *N/10* sodium hydroxid per 50 c. c. of medium, and the determination of the oxalic acid as oxalates as found in the same amount of medium.[2]

Oxalic acid was first precipitated as calcium oxalate, then dissolved in dilute hydrochloric acid and again precipitated.[3] It was redissolved in dilute sulphuric acid and titrated with standard potassium permanganate to a slight permanent pink.

The cultures were held two days at 30° C. and for the remaining period at 20°.

[1] Schiemann, Elisabeth. Mutationen bei aspergillus niger Van Tieghem. *In* Ztschr. Induk. Abstam. u. Vererbungslehre, Bd. 8, Heft 1/2, p. 1–35, 16 figs., 2 pl. (1 col.). 1912.

[2] The cultures were grown upon a modified Czapek's solution with the following composition:

Water	1,000 c. c.
Sodium nitrate	3 gm.
Potassium phosphate (KH_2PO_4)	1.0 gm.
Magnesium sulphate	0.25 gm.
Potassium chlorid	0.25 gm.
Ferrous sulphate	0.01 gm.
Cane sugar	50.0 gm.

[3] For a description of the method, see Currie, J. N., and Thom, Charles. An oxalic acid producing Penicillium. *In* Jour. Biol. Chem., v. 22, no. 2, p. 290. 1915.

TABLE I.—*Comparative oxalic-acid production in strains of Aspergillus niger and related organisms grown in flasks of 50 c. c. each of Czapek's solution containing 5 per cent of sugar*

[Acidity and oxalate radical expressed in cubic centimeters of *N*/10 sodium hydroxid required to neutralize 50 c. c. of medium]

Culture No.	Name of source.	7 days.		10 days.		14 days.		18 days.	
		Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.
142	*A. ficuum* (?)	116.17	116.21	142.85	155.82	167.90	182.90	153.21	170.01
2469.4	Soil, Delaware	97.57	48.76	103.15	94.35	111.88	96.59	99.53	99.02
111	*A. niger* (Amsterdam)	62.30	67.82	80.52	94.72	79.54	94.35	71.12	87.05
3528.7	Pittsburgh	103.74	48.57	97.96	79.95	89.92	85.56	61.14	60.34
4020.33	Soil, Texas	62.30	65.95	69.94	79.03	67.80	82.87	80.72	94.53
4049	Sardine paste	61.03	36.43	69.30	59.41	75.24	66.51	32.33	40.54
2657	Soil, England	29.78	30.64	58.78	74.73	29.58	45.59	22.92	39.42
4050	Chinese galls	50.16	28.58	54.36	51.00	51.12	47.64	37.32	44.09
2766	do	39.77	29.88	53.10	62.77	26.06	41.48	14.69	30.83
2774	Human ear	52.70	34.94	52.41	56.05	37.62	46.71	38.20	49.70
3534b	*A. cinnamomeus*	72.10	63.71	47.80	61.47	28.22	43.34	28.80	44.09
3547-354b	Soil, Kansas	47.80	45.96	50.74	65.20	58.78	74.92	34.68	54.93
2396	Missouri	46.24	31.01	45.55	56.05	33.90	48.95	15.28	31.39
2580	Pepper, Spain	81.11	84.07	49.57	63.90	32.90	48.20	33.50	49.66
4030.5	Unknown	34.97	11.96	28.60	17.51	13.90	15.32	2.35	16.44
3534c	*A. fuscus*	85.23	81.08	26.64	40.73	19.39	33.07	12.34	28.02
3522.30	*A. violaceo-fuscus* (?)	17.73	9.15	12.73	18.87	9.01	17.56	3.13	16.44
3534a	*A. niger allipes*	31.74	25.41	11.17	23.54	9.21	23.35	8.03	23.54
4030.1	*A. carbonarius*	31.54	14.57	5.58	18.31	6.66	21.33	3.53	13.85
4030.4	*A. ochraceus*	1.57	7.10	1.47	13.08	.78	14.70	.39	11.58

A study of Table I disposes effectually of the idea that because a species of Aspergillus is black or fuscous it must possess in specific measure the power to produce oxalic acid. It is noteworthy that all of the series possess this power in some degree and that some of the series show it in excessive degree. Others, however, produce this acid in no greater amounts than do members of other groups, as has been shown in the authors' previous paper.[1] These wide variations therefore indicate either a group of heterogeneous ancestry or a series of races of a single ancestry, which show great variation in the ability to produce a particular reaction. If such variation can be correlated with morphological characters, it is a valuable accessory in the identification of species. If cultures exhibit only quantitative differences in the reactions selected for study, such differences may be exceedingly important economically without justifying the description of separate species.

Nine forms representative of the range of variation found in Table I were selected for further experiment. No. 4047, growing upon strong lemon juice, was added. Tubes of Czapek's solution agar, Raulin's fluid, wort agar, and beef-peptone agar were prepared. Duplicate tubes of each of the four media were inoculated from a single tube of each of these 10 strains. The cultures were incubated at 37° C., until ripe spores were abundant, which was usually in about three days. Then transfers were made to fresh tubes of the same medium. In this way in a period of about five weeks each strain was transferred seven times upon each of

[1] Currie, J. N., and Thom, Charles. Op. cit.

these media and grown at 37°. At the end of that time they were reexamined for oxalic-acid production. The tabulated results are given in Table II, with the forms arranged in the same order as in Table I. The quantity of *N*/10 sodium hydroxid required to neutralize the free acid and the oxalate radical is shown in separate columns. The column marked "average" represents the average of the four previous figures.

TABLE II.—*Comparative oxalic-acid production of 10 strains of a black species of Aspergillus grown at 37° C., through seven transfers in parallel culture upon the media indicated*

[Acidity and oxalate radical expressed in cubic centimeters of *N*/10 sodium hydroxid required to neutralize 50 c. c. of Czapek's solution containing 5 per cent of cane sugar, after growth for 10 days]

Culture No.	Czapek's solution.		Raulin's solution.		Wort agar.		Beef-peptone agar.		Average.		Comparison of 10 days' growth from Table I.	
	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.	Acidity.	Oxalate.
142	165.18	190.62	150.89	192.58	120.57	135.30	125.57	142.22	143.05	165.23	142.85	155.82
2469.4	69.35	67.90	72.85	86.18	71.13	68.40	112.29	74.32	80.95	74.20	103.15	94.35
4047	69.77	46.66	71.04	56.78	53.70	39.76	65.80	56.54	65.08	49.93	.....	.....
111	82.49	98.76	84.52	100.98	73.76	89.88	83.51	100.98	81.07	97.65	80.52	94.72
4049	46.26	48.36	85.12	58.52	31.81	48.88	62.99	56.54	56.54	53.07	69.30	59.41
2766	33.15	40.74	80.50	107.66	49.85	67.40	43.27	56.30	51.69	68.02	53.10	62.77
2774	40.28	45.18	54.53	50.62	38.70	40.00	65.40	62.96	49.73	49.73	52.41	56.05
2580	53.39	65.92	73.45	77.28	50.86	67.16	38.23	54.32	53.98	66.14	49.57	63.90
3534c	28.14	45.18	23.95	39.76	63.02	55.30	22.04	35.56	34.29	43.95	26.64	40.73
4030.1	7.33	17.28	.....	11.16	21.48	11.16	4.43	15.80	11.08	13.22	5.58	18.31

A study of Table II shows a somewhat higher acid production for cultures propagated upon the Czapek's and Raulin's solutions than for those given upon wort and beef-peptone agar. In spite of occasional contrasting figures, the entire table fails to show any marked increase or decrease in acid-producing power by the treatment. This experiment tends to the conclusion that there are many strains or varieties of black *Aspergillus* spp. which differ markedly in the production of this reaction. These differences have persisted through many repetitions of the work with certain forms. Some of these forms, notably some of those producing the largest quantities of oxalic acid, have been in continuous culture by one of the writers for six years. Whether these strains would continue to produce oxalic acid in the same quantity if cultivated for a longer period is not known. The mutants described and distributed by Schiemann [1] (3534–a, 3534–b, and 3534–c) differ from each other in the quantity of acid produced. This difference is accompanied by a difference in color on the part of two of them, *A. fuscus* and *A. cinnamomeus*. In the case of *A. niger*, var. *altipes*, the strain as studied in this laboratory has lost the single morphological difference—long stalks—originally described; hence, at present it is not distinguishable from our Amsterdam

[1] Schiemann, Elisabeth. Op. cit.

stock culture (No. 111). This strain (No. 3534–a) is, however, the lowest oxalic-acid producer of all the strains closely related morphologically to the typical *A. niger*. These three forms, therefore, known to be closely related to each other and probably also to the Amsterdam culture (No. 111), differ markedly enough in this one reaction to suggest that the mutation which occurred was probably a quantitative readjustment among the enzyms.

In both tables some cultures show much more free acid, as indicated by titration, than is shown by the determination of oxalate. This difference, as redetermined for certain of these forms in the second experiment, is maintained in approximately the same relative proportion in Table II. It has held true also in experiments not included in this paper. No. 142, 111, 4020.33, 2657, 3547, 3546, 2580, 3534–a, 3534–c, and 3522.30 show slight differences between these determinations at 7 and 10 days. The other forms show much greater differences. As all the cultures become older, the oxalate determination equals, and even becomes greater than, the free-acid figure. Unfinished work to be reported later shows that citric acid forms part at least of the excess transiently found in the determination of free acid. Comparative study of the colonies themselves does not correlate these differences in acid production with morphology. The difference between the strains used appears, therefore, to be one of rate and quantity of reaction rather than a difference in kind of activity.

Although most of these forms had previously been studed carefully, a microscopic examination of each strain was made, in order to seek in morphology a possible basis for separation. The range of morphological characters found points to the existence of a series of closely related strains in which the differences are in measurement of parts, intensities of color, and quantitative differences in the production of particular reactions.

The members of this group grow under a wide range of cultural conditions. When colonies of a particular strain are grown simultaneously upon substrata of markedly different composition, distinct differences appear. All these strains, however, when grown under the same conditions, have so many common characters and so many intergradations that a group characterization upon the lines recently used by one of the writers for *Penicillium* spp.[1] will be more useful than any attempt to describe strains separately. This work harmonizes with the conclusion of Schiemann [2] that *A. niger* as commonly understood is an unstable or mutating group comparable to *Oenothera* spp.

[1] Thom, Charles. The Penicillium luteum-purpurogenum group. *In* Mycologia, v. 7, no. 3, p. 134-142, 1 fig. 1915.

[2] Schiemann, Elisabeth. Op. cit.

GROUP CHARACTERIZATION

COLONY CHARACTERS

Colonies spread rapidly upon Czapek's solution agar. They are at first white with abundant submerged mycelium and with more or less prostrate or trailing hyphæ radiating toward the periphery. Floccose aerial mycelium is occasionally developed later. Conidiophores arise as colorless branches from submerged or, more rarely, from aerial hyphæ and constitute the whole surface growth in most strains. With conidial production the colony color changes from white to fuscous black, but never shows any shade of green.[1] In certain forms Saito [2] records the color as passing through yellow to brownish black. The color found is rarely evenly distributed in the fruiting parts. Yellow or yellow-brown color may be present or absent in the upper 100μ of the stalk; in old cultures it is usually found in the vesicle and sterigmata, which frequently are deeply colored, even becoming brownish black or carbonaceous in certain races. Most of the color, as indicated by the researches of Linossier,[3] is deposited in the ridges or warts of the conidial wall. The submerged mycelium is uncolored in some forms, more or less deeply yellowed in others. The agar remains uncolored. All forms grow at 37° C. and, with the exception of *A. carbonarius*, are favored by that temperature.

MORPHOLOGY

STALK.—The stalks or conidiophores vary in thickness from 6 to 25μ, and in lengths from 0.5 to 10 mm. They are unseptate or indistinctly septate and have thick walls, smooth on the outside, and on the inside either smooth or, in some cases, with irregular thickenings. When broken these walls split lengthwise into strips. There is a great difference in the length of stalks in the same colony. In the denser center of the colony, developed upon fresh media with abundant food, the stalks are crowded together and shortest. At the margin the scattered fruiting heads are borne upon stalks which may be twice to several times the length of those in the center. If the center of the young colony be taken as typical of the race, the strains in culture fall roughly into three groups: (1) Short-stalked, with stalks 500 to 1,000μ; (2) intermediate, with stalks 1,000 to 3,000μ; (3) long-stalked, with stalks 3 to several millimeters in length. The first two groups, however, certainly shade into each other.

[1] Ridgway, Robert. Color Standards and Color Nomenclature. Pl. 46, 13''''. Washington, D. C., 1912.

[2] Saito, Kendo. Mikrobiologische Studien über die Zubereitung des Batatenbranntweines auf der Insel Hachijo (Japan). *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 1–3, p. 31. 1907.

[3] Linossier, Georges. Sur une hématine végétale, l'aspergilline. *In* Compt. Rend. Acad. Sci. [Paris], t. 112, no. 15, p. 807–808. 1891.

——— Sur une hématine végétale: l'aspergilline, pigment des spores de l'Aspergillus niger. *In* Compt. Rend. Acad. Sci. [Paris], t. 112, no. 9, p. 489–492. 1891.

HEADS.—Conidial heads vary exceedingly in size; the common forms show simultaneously mature heads varying from 100 to 400μ in diameter. This maximum becomes 1,000μ or more in the gigantic forms. The chains of conidia at first radiate uniformly, but as they lengthen they adhere into black masses or columns which separate more and more as size increases.

VESICLE.—The vesicle or enlarged apex of the stalk varies commonly from 20 to 50μ, but occasionally reaches 80 to 100μ; it is continuous with the lumen of the stalk, thick-walled, and with walls and often contents yellow to brown in age.

STERIGMATA.—The vesicle is fertile over its whole surface and bears sterigmata usually in two series. Examination of young or growing colonies commonly shows individual heads producing conidia upon sterigmata of the first series, other heads with both simple and branched sterigmata, and heads with well-differentiated primary sterigmata, each bearing its quota of three to several secondary sterigmata. Here, as elsewhere, the variations of measurement in the same colony destroy all faith in such figures as an exact means of separating forms. The classic descriptions of *A. niger* as summarized by Wehmer [1] give the primary sterigmata as 26 by 4 to 5μ, the secondary as 8 by 3μ. The secondary sterigmata vary perhaps within limits of 6 to 10μ by 2 to 4μ in the whole series, with 8 by 3μ as a fair average figure. The variation in primary sterigmata makes a length of 26μ an occasional average for slected heads only. Examination of many heads in some strains gives lengths of primary sterigmata averaging between 12 and 20μ with an occasional longer cluster and frequently much of this variation occurs within the individual head. In another group the maximum length lies between 20 and 30μ. Again, in a few strains, lengths of 40 to 60μ are seen, while two of this series in their largest heads show primary sterigmata up to 120μ in length, as given by Bainier [2] for *S. carbonaria*.

CONIDIA.—The formation of conidia in the *A. niger* series follows the process described by Thom [3] for *Penicillium* spp. This probably agrees with the process designated as endogenous by Bainier and Sartory [4] in that each conidium is first cut off from the conidial tube of the sterigma, then rounds itself up after secreting for itself a new wall, while the original cell wall is frequently distinguishable between the ripe conidia as a connective. Ripe conidia are subglobose, with, in most strains, a variation in the same culture of about 1μ in diameter; some run from 2.5 to 3.6μ,

[1] Wehmer, Carl. Die Pilzgattung Aspergillus in morphologischer, physiologischer und systematischer Beziehung unter besonderer Berücksichtigung der mitteleuropaeischen Species. V. Systematik. C. Schwarzbraune Arten. *In* Mém. Soc. Phys. et Hist. Nat. Genève, t. 33, pt. 2, no. 4, p. 103–107. 1901.

[2] Bainier, Georges. Sterigmatocystis et nematogonum. *In* Bul. Soc. Bot. France, t. 27 (s. 2, t. 2), p. 29–30. 1880.

[3] Thom, Charles. Conidium production in Penicillium. *In* Mycologia, v. 6, no. 4, p. 211–215, 1 fig. 1914.

[4] Bainier, Georges, and Sartory, Auguste. Etude d'un Aspergillus pathogène (Aspergillus fumigatoides n. sp.). *In* Bul. Soc. Mycol. France, t. 25, fasc. 2, p. 112. 1909.

others from 3 to 4μ, a few from 3 to 4.5μ, and in *A. carbonarius* from 6 to 10μ. Conidial walls are typically rough, with irregular, but usually oblong or barlike masses of coloring matter [the aspergillin of Linossier[1]] which run lengthwise of the conidial chain, and are absent at the ends of the spore or are replaced there by the connective when such is present. In some forms these bars are more or less completely broken up to form irregularly disposed rough tubercles. In experiments this coloring substance was dissolved by soaking in hot water, after which the original outer cell wall became visible. This experiment indicates that the difference in color between strains is due to the varying amount of aspergillin deposited between the outer, or primary, and inner, or true, spore walls. Upon careful study, *A. fuscus* (one of Schiemann's[2] mutants from *A. niger*) showed delicate traces of the typical bars of color; *A. cinnamomeus* with its much lighter color and smooth spores showed only diffused, not localized color.

Details in colonies of the same strain differ in successive cultures. These differences are nearly all quantitative, but they indicate great power of response to the stimulation of environment. The mutants, *A. cinnamomeus* and *A. fuscus*, separated by Schiemann,[2] differ from the usual form only in intensity of color, yet maintain these characters consistently in culture. *A. carbonarius* (4030.1) is a gigantic form in which the proportions are approximately quadrupled, while No. 2580 (*A. strychni*?) shows the same measurements except that the conidia remain with a maximum diameter of 4 to 4.5μ. Perhaps *A. niger* is a form comparable to *Oenothera* spp. in its tendency to produce mutants. There arise, therefore, a few forms with characters sufficiently tangible to separate by description. In a majority of the strains met with in culture, morphological differences are not sharp enough for diagnostic purposes. Nevertheless great and fairly stable differences in physiological activity are found among them. Two forms morphologically alike may thus differ greatly in economic importance.

NOMENCLATURE.—Current literature has accepted the name "*Aspergillus niger*" Van Tieghem,[3] for the black species of Aspergillus. *Sterigmatocystis autacustica* Cramer, obtained from a human ear, was undoubtedly one of the series of organisms, but was insufficiently described. *A. ficuum* (Reich.) P. Hennings, first named "*Ustilago ficuum*" by Reichardt in 1867, differs slightly in measurements, but the presumption of identity is based upon the constant occurrence of the

[1] Linossier, Georges. Sur une hématine végétale l'aspergilline. *In* Compt. Rend. Acad. Sci. [Paris], t. 112, no. 15, p. 807–808. 1891.

——— Sur une hématine végétale: l'aspergilline, pigment des spores de l'Aspergillus niger. *In* Compt. Rend. Acad. Sci. [Paris], t. 112, no. 9, p. 489–492. 1891.

[2] Schiemann, Elizabeth. Op. cit.

[3] Van Tieghem, P. E. L. Recherches pour servir à l'histoire physiologique des Mucédinées. Fermentation gallique. *In* Ann. Sci. Nat. Bot., s. 5, t. 8, no. 4, p. 240. 1867.

usual type of a black species of Aspergillus in practically pure culture in figs. *A. phoenicis* (Corda) Patouillard and Delacroix, recorded as *Ustilago phoenicis* by Corda[1] in 1840, differs also in the shape and smoothness of its conidia, although other data point to close relationship. Van Tieghem in his article discussed *A. nigrescens* of Robin (1848) and *A. nigricans* of Wreden (1867) and offered reasons for separating *A. niger* from these organisms, neither of which is adequately described.

The conspicuous character of these black colonies and their frequency in all sorts of decaying food make it difficult to believe that the species remained undescribed until 1867. A review of the literature of *Aspergillus* spp., and of those generic names used interchangeably with it by some of the earlier botanists, has included Micheli, Linnaeus, Sowerby, Persoon, Link, Ehrenberg, Fries, Greville, Corda, the Tulasnes, and Bonorden. Several names are found which might refer to this group, but are unaccompanied by either figures or descriptions which can be definitely shown to represent this species. The usage of Raulin[2] probably suggests the true explanation. The reference of *A. niger* to the genus Ascophora (syn. Rhizopus) by the French workers preceding Van Tieghem points to the conclusion that *A. niger* had been constantly confused with the mucors. The recognition by Robin, Cramer, and Wreden of black forms of *Aspergillus* spp. as the cause of mycotic diseases in the ear seems to have led directly to the recognition of the separateness of the black species of Aspergillus from the black mucors as a cosmopolitan organism.

The generic name "Sterigmatocystis," proposed by Cramer,[3] is based upon the assumption that in the forms of Aspergillus to which it was applied, there were always two sets of cells (basidia and sterigmata of some authors; primary and secondary sterigmata of Wilhelm and Wehmer) between the vesicle, or enlarged end of the conidiophore, and the actual conidial chains. This distinction was disregarded by Wilhelm,[4] reaffirmed by Eidam,[5] and again discarded by Wehmer.[6] Examination of thousands of cultures does not, in the opinion of the writers, justify the use of the separate generic name "Sterigmatocystis." There appears, therefore, no good reason to displace the name "*Aspergillus niger*" for at least a section of the group.

In a classification on color alone *A. cinnamomeus* and *A. fuscus* of Schiemann would be excluded from the series. If structure and meas-

[1] Corda, A. C. I. Icones Fungorum . . . t. 4, p. 9, pl. 3, fig. 26. Pragae, 1840.

[2] Raulin. Études chimiques sur la végétation des Mucédinées, particulièrement de l'Ascophora nigrans. *In* Compt. Rend. Acad. Sci. [Paris], t. 57, no. 4, p. 228–230. 1863.

[3] Cramer, Carl. Ueber eine neue Fadenpilzgattung: Sterigmatocystis. Cramer. *In* Vrtljschr. Naturf. Gesell. Zürich, Jahrg. 4, Heft 4, p. 325–337, pl. 2. 1859.

[4] Wilhelm, K. A. Beiträge zur Kentniss der Pilzgattung Aspergillus. 70 p. Strassburg-Berlin, 1877. Inaug. Diss.

[5] Eidam, Eduard. Zur Kenntniss der Entwicklung bei den Ascomyceten. *In* Beitr. Biol. Pflanz., Bd. 3, p. 377–433, pl. 19–23. 1883.

[6] Wehmer, Carl. Op. cit., p. 28, 34–35.

urement of conidial apparatus be used to define the group, certain strains of *A. ochraceus* Wilhelm and *A. wentii* Wehmer certainly fall within it. All of these forms are clearly related and may properly constitute a section of the genus Aspergillus without venturing a guess as to their genetic connection.

The group, therefore, may be held to consist of a series of forms, some of which seem to be connected so closely by intergrading forms as to make separation difficult if not impossible. Other members of the group notably represented by the mutations of Schiemann[1] show permanent and striking differences. *A. carbonarius* of Bainier may be a similar case. Upon morphology alone we may therefore be justified in retaining certain specific names as well-defined representatives of the sections of the group. The arrangement proposed begins with *A. nanus* Mont. for the diminutive form, *A. niger* Van Tieghem for the most numerously occurring section with primary sterigmata 20–30μ in length, *A. phoenicis* (Corda) Pat. and Delacr. with primary sterigmata about 50μ, *A. pulverulentus* McAlpine, or *A. strychni* Lindau, with very long sterigmata, and end our series with *A. carbonarius*, which has the long sterigmata and very large conidia.

In suggesting that the following names be retained as designating representative cultures falling within fairly well-defined sections of the group, it remains uncertain how many names may ultimately be required to designate forms permanently considered as species. The sections and citations follow:

I.—FORMS WITH SIMPLE STERIGMATA UP TO 20μ IN LENGTH

A. nanus Montagne, 1856, Syll. Gen. Spec. Crypt., p. 300, no. 1112. Saccardo, 1886, Syll. Fung. v. 4, p. 71.

IIa (BLACK OR BROWN).—FORMS WITH BOTH PRIMARY AND SECONDARY STERIGMATA; PRIMARY 20–30μ IN LENGTH

A. niger Van Tieghem, 1867, *in* Ann. Sci. Nat. Bot., s. 5, t. 8, no. 4, p. 240. As probable synonyms the following may be listed: *S. antacustica* Cramer, 1859, *in* Vrtljschr. Naturf. Gesell. Zürich, Jahrg. 4, Heft 4, p. 325; *A. echinosporus* Sorok., Paras.[2] p. 40, pl. 7, fig. 82–87. Ref. *in* Saccardo, 1895, Syll. Fung., v. 11, p. 592; *A. ficuum* (Reich.) Hennings, 1895, *in* Hedwigia, Bd. 34, Heft 2, p. 86; *A. fuliginosus* Peck, 1873, *in* Bul. Buffalo Soc. Nat. Sci., v. 1, p. 69; 1874, *in* 26th Ann. Rpt. N. Y. State Mus. Nat. Hist. [1872], p. 79; *A. nigrescens* Robin, 1853, Hist. Nat. Vég. Paras., p. 518, atlas, pl. 5, fig. 2; *A. nigricans* Wreden, 1867, *in* Compt. Rend. Acad. Sci. [Paris], t. 65, no. 9, p. 368. *A. phaeocephalus* (Durieu and Montagne), 1881, *in* Saccardo, Fungi Ital., fig. 903; 1886, *in* Saccardo Syll. Fung., v. 4, p. 76; *S. pseudonigra* Costantin and Lucet, 1903, *in* Bul. Soc. Mycol. France, t. 19, fasc. 1, p. 33–44; *A. ustilago* Beck, 1888, *in* Wawra. Itin. Princ. S. Coburgi, T. 2, p. 148; 1892, *in* Saccardo, Syll. Fung., v. 10, p. 526; *A. welwitschiae* (Bresadola) Henn.

[1] Schiemann, Elisabeth. Op. cit.

[2] Not seen by the author.

IIb.—FORMS DIFFERING FROM A. NIGER ONLY IN COLOR

A. cinnamomeus Schiemann, 1912, *in* Ztschr. Induk. Abstam. u. Vererbungslehre, Bd. 8, Heft ½, p. 1–35, 16 fig., 2 pl. (1 col.).

A. Schiemanni (Schiem.) Thom, n. comb. Syn. *A. fuscus* Schiem. (Schiemann, Elizabeth. Op. cit., 1912).

III.—FORMS WITH BOTH PRIMARY AND SECONDARY STERIGMATA; PRIMARY ABOUT 50μ IN LENGTH

A. phoenicis (Corda) Patouillard and Delacroix, 1891, *in* Bul. Soc. Mycol. France, t. 7, p. 118–120, pl. 9; syn. *Ustilago phoenicis* Corda, 1840, Icones Fung., t. 4, p. 9, pl. 3, fig. 26.

IV.—FORMS WITH BOTH PRIMARY AND SECONDARY STERIGMATA; PRIMARY UP TO 120μ IN LENGTH

A. pulverulentus McAlpine, 1896, *in* Agr. Gaz. N. S. Wales, v. 7, pt. 5, p. 302; probable synonym *A. strychni* Lindau, 1904, *in* Hedwigia, Bd. 43, Heft 5, p. 306–307.

V.—FORMS WITH BOTH PRIMARY AND SECONDARY STERIGMATA; PRIMARY UP TO 120μ IN LENGTH, SPORES DOUBLE SIZE

A. carbonarius (Bain.), Thom (Bainier, 1880, *in* Bul. Soc. Bot. France, t. 27 (s. 2, t. 2), p. 27–28).

The following list contains the names, original and often secondary citations, for the forms described as black or brown. Among these are some forms certainly not related to *A. niger*, but described in terms which might suggest such relationship unless critically examined. Wherever possible, the proper placing of the form is suggested. One original reference, that of Sorokin, has not been seen; all others have been critically examined. The forms are arranged alphabetically to species without reference to the describer's placing in Aspergillus or Sterigmatocystis.

S. antacustica Cramer (Cramer, Carl. Ueber eine neue Fadenpilzgattung: Sterigmatocystis. Cramer. *In* Vrtljschr. Naturf. Gesell. Zürich, Jahrg. 4, Heft 4, p. 325. 1859).

In the external ear of man; considered *A. niger* by Wilhelm (Wilhelm, K. A. Beiträge zur Kenntniss der Pilzgattung Aspergillus. 70 p. Strassburg-Berlin, 1877. Inaug. Diss.).

A. atropurpureus A. Zimm. (Zimmermann, A. Ueber einige an tropischen Kulturpflanzen beobachtete Pilze. II. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 8, No. 5, p. 218. 1902).

Not in the *A. niger* series; sterigmata and spores suggest the *A. glaucus* series.

A. batatae Saito (Saito, Kendo. Mikrobiologische Studien über die Zubereitung des Batatenbranntweines auf der Insel Hachijo (Japan). *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 1/3, p. 34. 1907).

Morphology given suggests *A. niger* in details, but in colony characters indicates a close relationship to *A. wentii*, Wehmer. This is confirmed by culture of a strain of *A. wentii* which showed this morphology.

A. brunneus Bain. (Bainier, Georges. Sterigmatocystis et nematogonum. *In* Bul. Soc. Bot. France, t. 27 (s. 2, t. 2), p. 29. 1880).

Colonies at first green, then black-brown; conidia 15 in diameter; probably *A. glaucus* series.

A. brunneus Delacr. (Delacroix, Georges. Espèces nouvelles de Champignons inférieurs. *In* Bul. Soc. Mycol. France, t. 7, p. 109, pl. 7. 1891).

Related to the preceding by color.

S. carbonaria Bain. (Bainier, Georges. Sterigmatocystis et nematogonum. *In* Bul. Soc. Bot. France, t. 27 (s. 2, t. 2), p. 27–28. 1880).

A culture from Dr. Blakeslee reproduces the morphology recorded by Bainier. A redescription of the form is therefore given.

A. carbonarius (Bainier), Thom., n. comb.

Colonies grown in Czapek's solution agar show vegetative mycelium white or with some yellow in submerged areas, broadly spreading, more or less zonate; sclerotia produced upon the surface of the substratum in old cultures; fruiting areas carbon-black; stalks colorless below, yellow to yellow-brown toward the apex, 4 to 6 mm. or longer and up to 25μ in diameter, with walls smooth, up to 4μ in thickness; heads globose varying in diameter up to 500μ, vesicles up to 90μ in diameter, fertile over entire surface, commonly with contents yellow-brown to black and in old heads forming with the primary sterigmata a hard brittle, carbonaceous mass; sterigmata in two series, primary sometimes 1-septate, from 20 to 40μ long in young or small heads and up to 120μ long in large heads, 5 to 13μ in diameter at the apex, secondary 8 to 14μ by 3 to 6μ; conidia at first smooth becoming rough when ripe, 5.5 to 10.5μ in diameter. Colonies grow well upon all culture media used, with temperature optimum below 37° C. Growth at 37° C. slow and more or less dwarfed.

A. cimmerius Berk. and Curtis (Berkeley, M. J. Notices of North American fungi. *In* Grevillea, v. 3, no. 27, p. 108, no. 656. 1875. Saccardo, P. A. Sylloge Fungorum . . . v. 4, p. 71. Patavii, 1886).

The color reference "aterrimus" and the spore size, elliptical 7μ, suggest *A. carbonarius*, but the data are inadequate.

A. cinnamomeus Schiem. (Schiemann, Elisabeth. Mutationen bei Aspergillus niger Van Tieghem. *In* Ztschr. Induk. Abstam. u. Vererbungslehre, Bd. 8, Heft 1/2, p. 1–35, 16 fig., 2 pl. (1 col.). 1912).

Differentiated by color and smoothness of spores, but maintains these distinctions uniformly. Obtained as a mutant from *A. niger* by Schiemann.)

A. cookeii (Cooke) Sacc. (Saccardo, P. A. Sylloge Fungorum . . . v. 4, p. 71. Patavii, 1886.)=*A. mucoroideus* Cooke (Cooke, M. C. Australian fungi. *In* Grevillea, v. 12, no. 61, p. 9. 1883).

Not recognizable from the description, but suggests a form of Syncephalastrum which we have had in culture.

A. echinosporus Sorok. (Sorok. Paras.[1] p. 40, pl. 7, fig. 82–87. Ref. in Saccardo, P. A. Sylloge Fungorum . . . v. 11, p. 592. Patavii, 1895).

The description suggests a Haplographium.

A. ficuum (Reich.) Henn. (Hennings, P. C. Ustilago Ficuum Reich.=Sterigmatocystis Ficuum (Reich.) P. Henn. *In* Hedwigia, Bd. 34, Heft 2, p. 86. 1895.)=*Ustilago ficuum* Reich. (Reichardt, H. W. Ein neuer Brandpilze. *In* Verhandl. K. K. Zool. Bot. Gesell. Wien, Bd. 17, p. 335. 1867).

Regarded as *A. niger* by Wehmer (Wehmer, Carl. Zur Kenntnis einiger Aspergillus-Arten. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 13/15, p. 394–395. 1907).

Slight differences in morphology are reported by Hennings, but disregarded by Wehmer.

[1] Not seen by the author.

A. fuliginosus Peck (Peck, C. H. Descriptions of new species of fungi. *In* Bul. Buffalo Soc. Nat. Sci., v. 1, p. 69. 1873. Peck, C. H. Report of the botanist. *In* 26th Ann. Rpt. N. Y. State Mus. Nat. Hist. [1872], p. 79. 1874).

No valid data are offered for separating this strain from *A. niger*.

A. fuscus Bonorden (Bonorden, H. F. Beiträge zur Mykologie. *In* Bot. Ztg., Jahrg. 19, no. 29, p. 202. 1861).

This is probably a very common form of Haplographium.

S. fusca Bain. (Bainier, Georges. Sterigmatocystis et nematogonum. *In* Bul. Soc. Bot. France, t. 27 (s. 2, t. 2), p. 29. 1880).

The morphology recorded differs from *A. niger* in the size of the conidia, "less than 9.4μ." The form has not yet been found by us.

A. fuscus Schiem. (Schiemann, Elisabeth. Mutationen bei Aspergillus niger van Tieghem. *In* Ztschr. Induk. Abstam. u. Vererbungslehre, Bd. 8, Heft 1/2, p. 1–35, 16 fig., 2 pl. (1 col.). 1912).

This mutant from *A. niger* is distinguished by the color and smoothness of its spores from the parent strain. The name of this and the preceding form are invalidated by the occurrence of the name *A. fuscus* Bonorden.

A. japonicus Saito (Saito, Kendo. Nachtrag zu der Abhandlung "Untersuchungen über die atmosphaerischen Pilzkeime, I." *In* Bot. Mag. [Tokyo], v. 20, no. 233, p. 61, 5 fig. 1906).

This species as described differs from *A. niger* in having but one set of sterigmata and showing granules at times upon the walls of the conidiophore. It is evidently very closely related to the regular forms of *A. niger*.

A. luchuensis (Inui, T. Untersuchungen über die niederen Organismen welche sich bei der Zubereitung des alkoholischen Getränkes. "Awamori" betheiligen. *In* Jour. Col. Sci. Imp. Univ. Tokyo, v. 15, pt. 3, p. 469, pl. 22, fig. 1–8. 1901).

This form differs from *A. niger* by showing green colors when young and by morphological details. It does not, therefore, belong in this series.

A. mucoroideus Cooke (Cooke, M. C. Australian fungi. *In* Grevillea, v. 12, no. 61, p. 9. 1883).

A. cookei Sacc.

A. mucoroideus Corda (Corda, A. C. I. Icones Fungorum . . . t. 2, p. 18, pl. 11, fig. 76. Pragae, 1838).

This is a different organism and not in this group. The description suggests *A. castaneus* Patterson.

A. nanus Montagne (Montagne, J. F. C. Sylloge Generum Specierumque Cryptogamarum . . . p. 300, no. 1112. Parisiis, 1856. Saccardo, P. A. Sylloge Fungorum . . . v. 4, p. 71. Patavii, 1886).

The description clearly distinguishes this organism as a member of the group and as having a single series of sterigmata about 15μ in length and spores 3μ in diameter.

A. niger Van Tieg. (Van Tieghem, P. E. L. Recherches pour servir à l'histoire physiologique des Mucédinées. Fermentation Gallique. *In* Ann. Sci. Nat. Bot., s. 5, t. 8, no. 4, p. 240. 1867.)=*S. nigra* Van Tieg. (Van Tieghem, P. E. L. Sur le développement de quelques Ascomycètes. *In* Bul. Soc. Bot. France, t. 24, p. 102–103. 1877).

Described by Van Tieghem as the active agent in fermenting tannin solutions.

A. nigrescens Robin (Robin, C. P. Histoire Naturelle des Végétaux Parasites . . . p. 518; atlas, pl. 5, fig. 2. Paris, 1853).

The organism of Robin has been called *A. niger* by Wilhelm (Wilhelm, K. A. Beiträge zur Kenntnis der Pilzgattung aspergillus. 70 p. Strassburg-Berlin, 1877. Inaug. Diss.), *A. fumigatus* by Siebenmann (Siebenmann, Friedrich.

Die Fadenpilze Aspergillus flavus, niger u. fumigatus; Eurotium repens (u. Aspergillus glaucus) und ihre Beziehungen zu Otomycosis Aspergillina. p. 82. Wiesbaden, 1883. Inaug. Diss.), and is judged undeterminable from the information given, by Wehmer (Wehmer, Carl. Zur Kenntnis einiger Aspergillus-Arten. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 13/15, p. 394–395. 3 fig. 1907). Robin's figures represent *A. fumigatus* much more closely than *A. niger*.

A. nigricans Wreden (Wreden, Robert. Recherches sur deux nouvelles espèces de végétaux parasites (Aspergillus flavescens et Aspergillus nigricans) de l'homme. *In* Compt. Rend. Acad. Sci. [Paris], t. 65, no. 9, p. 368. 1867).

This is regarded as *A. niger* by Wilhelm (Wilhelm, K. A. Beiträge zur Kenntniss der Pilzgattung Aspergillus. 70 p. Strassburg-Berlin, 1877. Inaug. Diss.) by Siebenmann (Siebenmann, Friedrich. Die Fadenpilze Aspergillus flavus, niger u. fumigatus; Eurotium repens (u. Aspergillus glaucus) und ihre Beziehungen zu Otomycosis Aspergillina. p. 82. Wiesbaden, 1883. Inaug. Diss.); and by Wehmer (Wehmer, Carl. Zur Kenntnis einiger Aspergillus-Arten, *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 13/15, p. 394–395. 1907).

A. nigricans Cooke (Cooke, M. C. Some remarkable moulds. *In* Jour. Quekett Micros. Club, s. 2, v. 2, no. 12, p. 140, pl. 9, fig. 3. 1885. Saccardo, P. A. Sylloge Fungorum . . . t. 4, p. 76. Patavii, 1886).

The occurrence in the human ear of forms known to be identical with *A. niger* makes a separate name unnecessary. The description of this organism corresponds with *A. nanus* Montagne, except that the spores are given as 5μ in diameter.

A. phaeocephalus Durieu and Montagne (Saccardo, P. A. Fungi Italici . . . fig. 903. Patavii, 1881. Saccardo, P. A. Sylloge Fungorum . . . v. 4, p. 76. Patavii, 1886).

No differences are given to warrant a separation of this form from *A. niger*.

S. phoenicis (Corda) Patouill. and Delacr. (Patouillard, Narcisse, and Delacroix, Georges. Sur une maladie des dattes produite par le Sterigmatocystis Phoenicis (Corda) Patouill. et Delacr. *In* Bul. Soc. Mycol. France, t. 7, p. 119, pl. 9. 1891.)=*Ustilago phoenicis* Corda (Corda, A. C. I. Icones Fungorum . . . t. 4, p. 9, pl. 3, fig. 26. 1840).

In describing the black Aspergillus as found upon dates, the authors compared this material to specimens in the museum labeled "*Ustilago phoenicis*" and attributed to Corda, thus establishing the identity of the organism of Corda, which was not recognizable from any previous references. *A. ustilago* Beck is described with the same measurements. The measurements of sterigmata place this form in the section of the group with primary sterigmata 50–60μ in length.

S. pseudo-nigra Costantin and Lucet (Costantin, and Lucet. Sur le Sterigmatocystis pseudonigra. *In* Bul. Soc. Mycol. France, t. 19, fasc. 1, p. 33–44. 1903).

This is regarded as *A. niger* by Wehmer (Wehmer, Carl. Zur Kenntnis einiger Aspergillus-Arten. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 13/15, p. 394–395. 1907).

The distinctions proposed by the authors are based upon pathogenicity and cultural reactions, not upon definite morphological characters.

S. pulverulenta McAlp. (McAlpine, Daniel. Australian fungi. *In* Agr. Gaz. N. S. Wales, v. 7, pt. 5, p. 302. 1896).

This is regarded as *A. niger* by Wehmer (Wehmer, Carl. Zur Kenntnis einiger Aspergillus-Arten. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 13/15, p. 394–395. 1907) after examining material received from McAlpine.

S. purpurea Van Tieg. (Van Tieghem, P. E. L. Sur le développement de quelques Ascomycètes. *In* Bul. Soc. Bot. France, t. 24, p. 101–103. 1877).

The information given is regarded as insufficient by Wehmer to separate this form (Wehmer, Carl. Die Pilzgattung Aspergillus in morphologischer und systematischer Beziehung unter besonderer Berücksichtigung der mitteleuropaeischen Species. V. Systematik C. Schwarzbraune Arten. *In* Mém. Soc. Phys. et Hist. Nat. Genève, t. 33, pt. 2, no. 4, p. 103–111. 1901).

A. purpureofuscus Fries (Fries, E. M. Systemata Mycologicum. v. 3, p. 388. Gryphiswaldae, 1829).

The hyphæ are described as *purpureofuscus*. This suggests certain of the *A. glaucus* series.

A. purpureofuscus Schw. (Schweinitz, L. D. von. Synopsis fungorum in America boreali media degentium. Secundum observationes. *In* Trans. Amer. Phil. Soc., n. s. v. 4, p. 282, no. 2680. 1834. Saccardo, P. A. Sylloge Fungorum . . . v. 4, p. 68, Patavii, 1886).

Not to be regarded as a member of this series from the description given.

A. Schiemanni (Schiemann) Thom, n. comb.=*A. fuscus* Schiemann (Schiemann, Elisabeth. Mutationen bei Aspergillus niger Van Tieghem. *In* Ztschr. Induk. Abstam. u. Vererbungslehre, Bd. 8, Heft 1/2, p. 1–35, 16 fig., 2 pl. (1 col.). 1912).

A new combination was necessary because the specific name *fuscus* had been already used by Bonorden for a species of Aspergillus and *fusca* by Bainier for a species of Sterigmatocystis. This form is distinguished only by its fuscous conidia which are smooth or very delicately roughened.

A. strychni Lindau (Lindau, Gustave. Aspergillus (Sterigmatocystis) strychni nov. spec. *In* Hedwigia, Bd. 43, Heft 5, p. 306–307. 1904).

This is regarded as A. niger by Wehmer (Wehmer, Carl. Zur Kenntnis einiger Aspergillus-Arten. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 13/15, p. 394–395. 1907).

The details of structure reported by Lindau for this form have been found in a culture (No. 2580) obtained in a study of red peppers (capsicum species) from Barcelona, Spain. The interior of these fruits had been destroyed and there remained a shell containing a mass of Aspergillus spores. The morphology obtained in cultures is identical with that of *A. carbonarius*, as described above, except that the conidia remain 4μ in diameter, as in the ordinary form of *A. niger*.

A. ustilago Beck (Wawra, Heinrich. Itinera Principum S. Coburgi. T. 2, p. 148. Wien, 1888. Saccardo, P. A. Sylloge Fungorum . . . v. 10, p. 526. Patavii, 1892).

The description relates this form to the section of the *A. niger* group showing sterigmata 50μ or more in length.

A. violaceo-fuscus Gasper. (Gasperini, G. Sopra un nuovo morbo che attacca i limoni e sopra alcuni ifomiceti. *In* Atti Soc. Toscana Sci. Nat. Pisa, Mem., v. 8, fasc. 2, p. 326. 1887).

The conidia are reported as ovoid, 3 to 6 by 3 to 3.5μ. This form probably belongs elsewhere.

A. welwitschiae (Bresadola) Hennings.

This is regarded by Wehmer as *A. niger* (Wehmer, Carl. Zur Kenntnis einiger Aspergillus-Arten. *In* Centbl. Bakt. [etc.], Abt. 2, Bd. 18, No. 13/15, p. 394–395. 1907).

No data are reported which warrant the establishment of a separate species.

SOME EFFECTS OF THE BLACKROT FUNGUS, SPHAEROPSIS MALORUM, UPON THE CHEMICAL COMPOSITION OF THE APPLE[1]

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INTRODUCTION

Various investigators have concerned themselves with the taxonomy, morphology, and life history of *Sphaeropsis malorum* Peck. This fungus occurs upon the twigs and branches of the apple (*Malus sylvestris*), producing cankers (15);[2] upon the foliage, where it produces a characteristic leaf-spot (22, 24); and upon the fruits, where its growth results in a decay that is unique because the diseased tissues undergo a rapid and characteristic blackening, because there is neither softening nor breaking down of the affected areas, and because loss of water and consequent shriveling do not begin until some time after the entire fruit has become involved. The occurrence of the disease upon the fruits of the apple appears to be very much less common in the North and East than in the South, where it frequently destroys 25 to 50 per cent of the crop (27).

The fact that the organism is able to attack tissues which vary so widely in chemical composition indicates that a study of its physiology should yield results of considerable interest. Very little attention has thus far been paid to the nature and extent of the chemical changes produced in the fruit by this organism, and it was to obtain information in regard to these changes that the present study was undertaken.

The present paper presents the results of a quantitative analytical study of the chemical composition of normal mature apples in comparison with others of the same variety attacked by *Sphaeropsis malorum*. The work was done in the laboratories of plant physiology of the Alabama Polytechnic Institute and Agricultural Experiment Station in the summer and autumn of 1915. The problem was suggested to Messrs. Foster and Culpepper, who were at that time graduate assistants in the laboratory, by Dr. Caldwell, who outlined the general methods of study to be employed and supervised the work. The major portion of the analytical work was done by Messrs. Foster and Culpepper. Upon their removal to other laboratories at the end of the summer, certain portions of the analyses were necessarily left incomplete; these have been completed,

[1] Published with the permission of the Director of the Alabama Agricultural Experiment Station.

[2] Reference is made by number to "Literature cited," p. 39-40.

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some portions of the work have been repeated, and the results have been prepared for publication by Dr. Caldwell, who is responsible for the conclusions drawn from the analytical data.

ANALYTICAL METHODS

The analytical methods employed are based upon those devised by Waldemar Koch and his pupils (9, 10, 11, 12) for use in the quantitative analysis of animal tissues. With the subsequent application of these methods to the study of plant tissues, a considerable number of modifications of the original scheme of analysis have been found necessary, and these have been incorporated into the analytical procedure. For some of these we are indebted to Dr. Fred C. Koch or to workers in his laboratory. Others of them were worked out by the writers in the course of this and similar investigations. A number of determinations of individual constituents have also been made by the employment of special methods, so that the results reported are believed to present a rather complete statement of the chemical differences between sound and diseased fruits.

The apples used in the experiments were of the Red Astrachan variety and were grown in the orchards of the Alabama Agricultural Experiment Station. The various lots employed in the work were gathered between June 17 and June 23, all those employed in any one series of analyses having been collected at one time and from the same tree. At the earliest date mentioned, the sound fruits were fully mature but had not begun to soften. As all the trees of this variety showed very severe blackrot infection, it was possible to secure at any desired time and from a single tree specimens showing any desired stage of the disease, together with sound fruits of the same age.

The procedure employed in collecting samples was the same in every case; a considerable number of wholly sound fruits, an equal number of fruits, each of which was approximately half-decayed, and a third lot, all of which were entirely decayed, were picked from the trees and carried at once to the laboratory. A careful examination of each fruit was then made in order to be sure that the diseased apples were free from organisms other than *Sphaeropsis malorum*, and three lots of material were prepared. The first lot, designated throughout this paper as "normal," was made up of selected portions of perfectly sound, normal fruits; the second lot consisted of decayed portions of fruits, one-half or three-fifths of which were invaded by blackrot, and is here termed "half-decayed"; the third lot, made up of fruits in which decay was complete, is designated "wholly decayed." Each lot of material was ground to a pulp by passing it through a food chopper and completing the grinding in a mortar. A portion was then removed, accurately weighed in a previously dried and tared crucible, and employed in a moisture determina-

tion. From the remainder, 100 gm. were weighed out, transferred to a closely stoppered flask, and preserved in such a quantity of redistilled 95 per cent alcohol as sufficed to give an alcohol concentration of 85 to 87 per cent. As the moisture content ranged from 87.39 per cent in the sound fruits to 86.14 per cent in completely decayed apples, each 100-gm. sample received 830 to 860 c. c. of alcohol. Preliminary moisture determinations had been made in order to ascertain the approximate water content. The whole operation of pulping, weighing samples, and transferring to the alcohol could be carried out in 8 to 10 minutes, so that little opportunity was offered for chemical changes resulting from exposure of the ground tissues to the air. The material was allowed to stand in the alcohol for seven days, with frequent shaking, and was occasionally warmed on a water bath to hasten the coagulation of the proteins and the extraction of constituents readily soluble in alcohol. The analyses of any given sample of material were begun on the eighth day after it had been collected.

The initial procedure in the method of analysis employed consists of successive extractions of the material with alcohol, ether, water, and alcohol, which divides the material into two portions, a portion consisting of the constituents extracted by these solvents and designated, for a reason which will presently appear, as "Fractions 1 and 2," and an insoluble residue designated "Fraction 3." The details of the method of extraction follow.

The extractions were carried out in an apparatus originally designed for the analysis of rubber insulating materials (6). This apparatus has very great advantages over the ordinary Soxhlet apparatus in that it has only two glass parts, a small Erlenmeyer extraction flask and an extraction cup bearing a side-arm siphon. The condenser, which is made of block tin, fits within the neck of the flask and bears a flange which forms a perfect seal, so that the dropping of cold water from the condenser upon the flank is prevented. The extraction cup is suspended from the condenser at such a height that the bottom is just above the level of the boiling solvent; hence, the material is kept constantly at a temperature equal to that of the liquid, which usually boils vigorously in the cup. The absence of ground-glass connections obviates the danger of loss from breakage, while the compact character of the apparatus permits the carrying out of a large number of extractions simultaneously upon an ordinary hot plate or water bath.

The extraction was begun by transferring the preserved material to previously dried and weighed Schleicher and Schüll extraction thimbles, in which all the subsequent extractions were made. Three thimbles of the size designated by the makers as "Hülsen für Extractions-apparate Nr. 603, 30×80 mm.," with the upper 20 mm. cut off, usually sufficed to contain a sample of 100 gm. In filling, the thimbles were placed in the glass siphon cups, which were then set into funnels dripping into

flasks, and the material was transferred by filtering the alcohol used for preservation repeatedly through the thimbles until it had been freed from solid particles. As the alcohol used in preserving the samples contained such constituents as were readily soluble in cold alcohol, it constituted a cold extract and was preserved for addition to the products of the subsequent extractions.

The extraction flasks were prepared by placing about 75 c. c. of redistilled 95 per cent alcohol in each, fitting the condensers, and warming gently on an asbestos plate over a gas flame. As soon as all dripping from the filled extraction cups had ceased, the material in each was pressed down with a glass rod, a weighed circle of ashless filter paper was fitted carefully over the top to prevent loss of finer particles, the cups were suspended from the condensers, and the flame so regulated that the filling and emptying of the siphon cups occurred about three times per minute. The extraction was continued for 12 hours; but at intervals of 3 to 4 hours the flame was turned out, the solutions collected from the extraction flasks, and equal quantities of 95 per cent alcohol introduced. This procedure has the twofold advantage that it removes the possibility of alteration in the dissolved materials by prolonged boiling in alcohol, while it at the same time prevents the slowing down of the extraction through an elevation of the boiling point of the solvent.

At the close of the alcohol extraction, the flasks were emptied, washed out with boiling 95 per cent alcohol, the material in the cups was pressed with a rod to remove the alcohol as completely as possible, and the extracts and washings combined and preserved. The extraction flasks now received 75 c. c. of ether each, and a 12-hour extraction with ether was made. While ether removes only very small quantities of material, which, except in the case of very heavily cutinized tissues, is quantitatively removed in the first two hours of the extraction, long-continued treatment with boiling ether greatly facilitates subsequent comminution of the tissues.

At the end of the ether extraction the material was warmed to drive off the ether, removed from the cups to a mortar, and ground into a fine powder. Bits of cuticle and fragments of vascular tissue were cut into fine bits with scissors and separately ground. The powdered material was then transferred to a stoppered flask with 100 c. c. of distilled water, placed on a boiling water bath, and heated with frequent shaking for 12 hours. Enough 95 per cent alcohol was then added to make the concentration of the solution 85 per cent, and the heating on the water bath was continued for a second 12-hour period. The solids were then collected into the original extraction thimbles by filtering the solution repeatedly through them, and a final alcohol extraction of 12 hours' duration was made. The residue remaining after this extraction constituted the alcohol-ether-water-insoluble fraction 3. It was dried to constant weight in the extraction thimbles, an aliquot part taken for

duplicate or triplicate ash determinations, and the remainder preserved in a desiccator for subsequent analysis in the manner to be presently described.

The soluble portions of the material were now contained in the alcohol originally employed for the preservation of the material, in that used for the first extraction and for washing the flasks, in the water-alcohol mixture in which the powdered material had been heated after extraction with ether, and in the ether extract. This last was now heated on a water bath until nearly all the ether had been driven off, when it was taken up with hot 70 per cent alcohol. All the solutions were then combined, whereupon some precipitation occurred. The precipitate was brought again into solution by warming the flask and adding sufficient boiling water to reduce the alcohol concentration to 70 per cent. When a perfect solution had been secured, the solution was transferred to a volumetric flask, made up to 2,000 c. c. with 70 per cent alcohol, and 200 c. c. were removed for the determination of the total solids. The remainder of the solution was transferred to beakers, placed upon a water bath kept at 75° C., and evaporated down to a sirupy consistency, a little absolute alcohol being added from time to time. The material was finally allowed to become alcohol free and was then taken up with sufficient distilled water, in most cases about 700 c. c., to form a perfect emulsion, and transferred to a 1,000 c. c. volumetric flask. Twenty c. c. of chloroform were added, the flask was vigorously shaken for several minutes, and the shaking was continued while 10 c. c. of concentrated hydrochloric acid were very gradually added. The flask was then filled to the mark with distilled water, stoppered, shaken at short intervals for 2 hours, and finally submerged to the neck in cold running water for 48 hours. At the end of this time the solution was clear, the lipoids having been partially carried down by the chloroform layer, while a part adhered to the walls and neck of the flask.

The solution was now filtered through a dry filter, great care being taken to prevent the transfer of the chloroform layer or the precipitated material upon the flask walls to the filter paper. After the filter had been allowed to drain well, the volume of the filtrate was noted. It constitutes the water-soluble portion of the alcohol-ether-water extract, and is designated as fraction 2.

The material precipitated by the chloroform, together with that held in the chloroform layer, constituted the lipoid precipitate, fraction 1. It was next collected by thoroughly washing the filter paper and the neck and walls of the precipitation flask with a large volume of boiling 95 per cent alcohol from a wash bottle, 500 to 600 c. c. being necessary with most samples. When a uniform solution had been secured, the flask was transferred to the water bath and kept at 70° C. until the chloroform had been completely driven off. The flask was then filled to the mark with warm 95 per cent alcohol, and aliquot parts of the

solution were at once taken for determinations of dry weight, phosphorus, nitrogen, carbohydrates as reducing sugars before and after hydrolysis, and ash.

The following determinations were then made upon fractions 1 and 2: Dry weight, ash, total phosphorus, total nitrogen, ammonia (in fraction 2), carbohydrates (as reducing sugar before and as invert sugar after acid hydrolysis in fraction 2; as hydrolyzable polysaccharids in fraction 3), and total acids (in fraction 2). Details of the determinations follow.

DRY WEIGHT.—The dry weight was ascertained in the case of fractions 1 and 2 by taking an aliquot part of the solution, evaporating to constant weight in a previously weighed platinum or porcelain dish at 102° to 105° C., weighing, and calculating the total weight from the results. Fraction 3 was weighed entire after drying to constant weight in the tared diffusion thimbles in which the extraction had been carried out.

ASH.—Ash was determined upon the portions of fractions 1 and 2 which had been taken for dry-weight determinations, and upon an aliquot of fraction 3. The procedure was the same in all cases. A preliminary test determined the presence or absence of free acids; if such acids were present, the sample was neutralized with sodium hydroxid and a correction for the sodium chlorid thus formed was made in the final weighings. The samples for ashing were transferred to previously dried, weighed porcelain crucibles, dried to constant weight in the oven, weighed, then placed within larger nickel crucibles. The contents were very gradually charred over a low flame, and finally burned to constant weight, averages of duplicate or triplicate samples being taken as true readings.

TOTAL PHOSPHORUS.—Determinations were made by the Neumann-Pemberton method as described by Plimmer (16, p. 543) and by Mathews (13, p. 893–895). The method consists essentially in the conversion of organic to inorganic phosphates by the addition of concentrated sulphuric acid and nitric acid, conversion of the phosphates into ammonium phosphomolybdate, dissolving the phosphomolybdate in a known excess of sodium hydroxid, and titrating the reduction in alkalinity of the sodium hydroxid with sulphuric acid. Determinations of inorganic phosphorus were attempted, employing the usual methods, but the amounts were so small—in no case amounting to 4 mgm.—that separate determinations were abandoned.

TOTAL NITROGEN.—Nitrogen was determined by the employment of the Gunning-Arnold modification of the Kjeldahl method. By reason of the very small amount of nitrogen present in fraction 2, the original intention to separate the nitrogenous constituents of the fraction into proteoses, peptones, polypeptids, amino acids, and nitrogen bases by separate determinations was abandoned. Accuracy in such determinations would have necessitated the extraction of very large amounts of material, which was impossible in the limited time available for the work.

Ammonia determinations were made in all cases upon fraction 2 by aerating the alkaline solution, passing the escaping air through an absorption tube containing *N/10* sulphuric acid, titrating the acid, and estimating the ammonia from the decrease in acid strength. In the case of both normal and diseased fruits, fraction 1 gave by the Kjeldahl method only slight traces of nitrogen, and the figures are consequently omitted from the tables.

CARBOHYDRATES.—In the case of fraction 2, the portion taken for sugar estimation was freed from organic acids and tannins by adding normal lead acetate in excess, diluting, filtering, removing the excess of lead by the addition of saturated sodium-sulphate solution, and again filtering (26, p. 43). An aliquot portion of the neutral solution was then taken for determination of reducing sugar by the Bertrand volumetric method (16, p. 228–229) and the amount of sugar calculated as dextrose by the use of the Munson and Walker tables (26). Total sugars were determined by the same methods upon a second portion of the solution, after inversion by heating for 10 minutes in a water bath kept at 70° with 3.5 per cent of hydrochloric acid and subsequent neutralization of the cooled solution. Total sugars were estimated as invert sugar, and the disaccharids were determined by a difference of readings before and after inversion.

The polysaccharids in fraction 3 were estimated as dextrose, by five hours hydrolysis with 2.5 per cent of hydrochloric acid under a reflux condenser, neutralizing the cooled solution, clearing of nonsugars with lead acetate, filtering, and employing the Bertrand method. No attempt was made to secure a quantitative separation of the mixture of sugars resulting from the hydrolysis into its various constituents. It is probable that the figures are low and that longer hydrolysis would have slightly increased the yield. The figures given, however, afford a safe basis for such general comparisons of the chemical composition of normal and diseased fruits as it is the purpose of the writers to make.

Very considerable difficulties were encountered in attempting to estimate the sugars in the lipoid precipitate, fraction 3. In the analytical scheme originally developed by Koch and his coworkers (10), sugars in this fraction are determined by evaporating a portion of the solution to an alcohol-free paste, taking up with water, hydrolyzing for 24 hours with 4 per cent of hydrochloric acid under a reflux condenser, concentrating the solution, drawing off the aqueous layer through a separatory funnel, clearing the solution with anhydrous sodium sulphate, and making a determination of sugar by the Bertrand method. The lipoid precipitate obtained from plant tissues contains true fats, waxes, lecithins, chlorophyll and derived or associated pigments, tannins and their derivatives, and resins. In the course of a 24-hour hydrolysis very considerable changes occur in some of these constituents, with the production of compounds which reduce Fehling's solution. Glycerol, which is absent

before the hydrolysis, is present, apparently as a result of the hydrolysis of some lipoid substance in the presence of hydrochloric acid as a catalyst. The glycerol can not be quantitatively removed without the removal of some sugar. Precipitation with normal lead acetate, with ferric salts, or with other ordinarily used precipitants does not completely free the hydrolysate from nonsugar constituents which combine with the copper of Fehling's solution. In consequence, the determinations for sugar in this fraction are uniformly high. While devoid of absolute quantitative value, they were made under identical conditions and, hence, have a certain comparative value.

RESULTS OF THE ANALYSES

COMPOSITION OF THE FRUITS USED

Prior to the discussion of the analytical results, it is pertinent to present data upon the composition of the apples used, which differ somewhat widely from those given by other investigators. The only analyses of Red Astrachan apples found in the literature are those made by Browne (1, 2) and Jones and Colver (7). Browne analyzed fruit grown at State College, Pa., while Jones and Colver analyzed two samples, one grown under irrigation and the other without irrigation, from unknown localities in Idaho. The data presented in Table I show that the apples employed in this work were somewhat lower in solids and reducing sugar, while higher in acid, in disaccharids expressed as cane sugar, and in insoluble residue than were those analyzed by the authors named. These differences are due in part to differences in the degree of maturity of the fruits used, in part to the very different climatic conditions under which the samples were grown, and in part to the use of varying methods of analysis. The apples used were taken prior to the beginning of the rapid digestion of carbohydrates attendant upon ripening, as attested by the presence of small quantities of starch, and the differences between normal and decayed fruits which appear in the results are those consequent upon the activities of the fungus.

MOISTURE LOSSES CONSEQUENT UPON ATTACK BY THE FUNGUS

The moisture content of the normal and the diseased fruits taken for analysis was determined by drying weighed portions of the finely ground and uniformly mixed masses of pulp, from which samples for analysis were subsequently taken, in an oven at 105° C. until constant weight was obtained. The writers are aware that the use of this method involves some error as a result of the decomposition of the sugars of the fruit, particularly of the levulose, as has been shown by Browne (2), but the fact that no vacuum drying apparatus was available rendered its use imperative.

In order to gain some idea of the magnitude of the constant and increasingly rapid loss of weight which accompanies decay, a number of

fruits showing just perceptible initial areas of tissue attacked by *Sphaeropsis malorum* were removed from the tree, weighed, the stems carefully sealed with paraffin, the increase in weight due to this treatment noted, and the fruits kept under observation. They were again weighed when decay had involved approximately one-half the tissues of each fruit, and again as soon as decay had become complete. The weight of the normal fruits was 566 gm.; when half-decayed, this had been reduced to 559 gm., or 98.76 per cent the original weight; when wholly decayed the weight was 539.6 gm., or 95.39 per cent of the original weight. Inasmuch as the moisture contents of the normal, half-decayed, and wholly decayed tissues taken for analysis were 87.39 per cent, 86.62 per cent, and 86.14 per cent, respectively, of the total weights, it is obvious that the loss in weight is due principally to other causes than the escape of water, partial proof of which is given by the fact that in the apples from which these weights were obtained the stems had been sealed, while the peel of every fruit remained intact.

TABLE I.—*Composition of normal mature fruit of Red Astrachan apple*

Analyst.	Source of fruit.	Total solids.	Ash.	Acidity as malic.	Crude fiber.	Reducing sugar.	Cane sugar.	Protein.
		Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Browne (1).....	State College, Pa.	15.30	0.37	1.038	.....	6.67	3.53	.....
Jones and Colver (7)	Nonirrigated orchard, Idaho.	18.10	.....	.9457	.....	6.98	2.15	0.288
Do.....	Irrigated orchard, Idaho.	14.73	.....	.890	.....	6.08	2.91	.560
Culpepper, Foster, and Caldwell.	Auburn, Ala.....	12.94	.2548	.9288	2.10	.574	4.960	.245

A comparison of the transpiration of normal apples given similar treatment with that of decaying apples would lead one into error, since the release of energy resulting from decomposition would manifestly increase transpirational water loss from the decaying fruits. While losses of weight due to transpiration and to respiration consequently can not be separately measured, it is clear from a comparison of the figures given that the primary and chief cause of loss in weight must be accelerated respiration. For these reasons, the analytical data now to be presented are in all cases based upon 100 gm. of fresh normal tissue, and have been corrected to this basis by the division of all figures for half-decayed fruit by 1.01255, of all figures for completely decayed material by 1.0492. These ratios are based upon the figures for losses in weight during decay obtained in the experiment just described, and the corrected figures are regarded as very close approximations to equal amounts of normal tissue, especially since the fruits employed in any one group of analyses were carefully selected for equal size and degree of maturity. Table II presents in tabular form a summary of the results of the analyses.

TABLE II.—*Composition of 100 gm. of Red Astrachan apples, normal and mature, half-decayed as a result of attack by Sphaeropsis malorum, and wholly decayed as a result of attack by Sphaeropsis malorum*

NORMAL AND MATURE

Fraction.	Dry weight.		Phosphorus.		Nitrogen.		Ammonia.
	Actual.	Percentage of total weight.	Actual weight.	Percentage of total phosphorus.	Actual weight.	Percentage of total nitrogen.	
	Gm.		*Gm.*		*Gm.*		*Gm.*
1	0.624	4.82	0.0101	30.25	Trace.	0	.....
2	10.216	78.94	.0191	57.22	0.0210	34.82	0.00375
3	2.100	16.24	.00418	12.53	.0393	65.17	.....
Total	12.940	.....	.03338	.....	.0603	.....	.....

HALF-DECAYED

1	0.519	4.26	0.0097	27.40	Trace.	0	.....
2	9.396	75.94	.0237	66.48	0.01659	26.79	0.003621
3	2.458	19.80	.00217	6.12	.0443	73.21	.....
Total	12.373	.....	.03557	.....	.06089	.....	.....

WHOLLY DECAYED

1	0.758	6.29	0.009055	25.00	Trace.	0	.....
2	7.857	65.14	.0200	57.89	0.00933	16.39	0.003485
3	3.445	28.57	.00714	17.11	.0476	83.16	.....
Total	12.060	.....	.03619	.....	.05693	.....	.....

NORMAL AND MATURE

Fraction.	Ash.		Carbohydrates.			Hydrolyzable polysaccharids.		
	Actual weight.	Percentage of total ash.	Lipoid sugars.	Reducing sugars.	Disaccharids.	Starch.	Other than starch.	Total.
	Gm.		*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
1	0.0017	0.66	0.4797	.....	.....	.....	.....	.....
2	.175	68.22	.....	0.574	4.960	.....	.....	.....
3	.0798	31.11	.....	.....	.....	0.321	0.315	0.636
Total	.2565	.....	.....	.....	.....	.....	.....	.....

HALF-DECAYED

1	0.0014	0.533	0.02488	.....	.....	.....	.....	.....
2	.2050	78.66	.....	0.1619	3.051	.....	.....	.....
3	.0542	21.81	.....	.....	.....	0.344	0.771	1.115
Total	.2606	.....	.....	.....	.....	.....	.....	.....

WHOLLY DECAYED

1	Trace.	0	0.1269	.....	.....	.....	.....	.....
2	0.277	85.11	.....	0.0609	2.234	.....	.....	.....
3	.0482	14.89	.....	.....	.....	0.318	1.086	1.404
Total	.3252	.....	.....	.....	.....	.....	.....	.....

The most immediately obvious fact observed upon inspection of Table II is the very considerable decrease in the total dry weight of solids recovered in the analyses of partially and totally decayed fruits. In comparison with a total dry weight of 12.940 gm. in normal fruits, the half-decayed material had 12.373 gm., a loss of 0.567 gm., or 4.38 per cent, while completely decayed material had a dry weight of 12.060 gm., with a loss of 0.880 gm., or 6.80 per cent. When the distribution of the dry weight between the three fractions is considered, it is apparent that the progress of the disease is accompanied by a very marked decrease in the constituents soluble in alcohol, ether, or water and consequently recovered in fraction 2. These make up 78.94 per cent of the dry weight of the normal fruit, 75.94 per cent of the weight of the half-decayed fruit, and 65.14 per cent of the weight of the wholly decayed material, a total decrease of 13.85 per cent. This reduction in fraction 2 is accompanied by a decrease, followed by an increase, in the lipoid material constituting fraction 1, and by a steady increase in the quantity of insoluble residue (fraction 3). In half-decayed fruits, lipoids make up 4.26 per cent and in wholly decayed fruits 6.29 per cent of the total dry weight, as compared with 4.82 per cent in normal tissues. There is consequently an absolute increase of 30.7 per cent in the lipoids as a result of the diseased condition. Concurrently, there is in the insoluble constituents making up fraction 3 an increase from 16.24 per cent in sound apples to 19.80 per cent in half-decayed fruit and 28.57 per cent in wholly decayed fruits. With a total dry weight equaling 12.060 gm. for wholly decayed fruits, the insoluble residue totals 3.445 gm., while a total dry weight of 12.940 gm. of sound fruit has an insoluble residue of only 2.100 gm. Stated in summary fashion, the result of the progress of disease to complete decay of the tissues is an increase of the insoluble residue to 176.23 per cent and of the lipoid fraction to 130.7 per cent of the normal, with a concurrent decrease of the water-soluble portion of the alcohol-ether-water extract to 82.29 per cent of normal. The significance of these differences will appear as the details of the analysis are discussed.

The nitrogenous constituents are practically wholly contained in fractions 2 and 3, since fraction 1, both in normal and in decayed fruits, uniformly gave amounts too small to be included in the tables. The total amounts of nitrogen found are very small, and there is a slight decrease, amounting to only 3.4 mgm. in an original total of 60.3 mgm., in the completely decayed fruit. The significant feature of the results lies in the fact that there is a steady decrease in the nitrogen of fraction 2, which represents proteoses, peptones, polypeptids, amino acids, and nitrogen bases, as the disease progresses. The amounts in this fraction are 21, 16.59, and 9.33 mgm. for sound, half-decayed, and completely decayed fruits, respectively. There is a corresponding, though not absolutely compensatory, increase in the nitrogen of fraction 3. The figures for that fraction

are 39.3, 44.3, and 47.6 mgm., and since these amounts may fairly be considered as the nitrogen of proteins, the employment of the factor 6.25 gives 245.6, 276.8, and 297.5 mgm. of protein for the three lots of tissue, or a gain of 51.9 mgm. of protein in the course of the process of decay. This unquestionably represents the synthesis of protein by the attacking fungus. It may be further pointed out that the gain in nitrogen in fraction 3 is not sufficient to account for the decrease in fraction 2; only about two-thirds of the 11.67 mgm. lost from fraction 2 by completely decayed tissues is represented by the gain of 8.3 mgm. occurring in fraction 3, and the total nitrogen figures are 60.3 mgm. for sound and 56.93 mgm. for completely decayed fruits, indicating a slight loss of nitrogen. Determinations of ammonia made upon aliquot parts of fraction 2 immediately upon completion of the separation showed a slight decrease in the amount present as the disease progressed; for sound tissue the figures were 3.757 mgm. of ammonia; for partially decayed tissue, 3.620 mgm.; for entirely decayed tissue, 3.485 mgm. Although these differences are small and subject to a relatively large experimental error, as must always be the case when extremely low concentrations of ammonia are determined, they nevertheless are concordant and compel the conclusion that the peptone and amino nitrogen of the fruit are steadily decreased with the progress of the disease by two processes, one a degradation process which results in the formation and escape of ammonia, the other an anabolic process which converts the soluble nitrogenous constitutents into insoluble forms, presumably within the cells of the invading organism. Reed and Stahl (21) reported the presence of erepsin in cultures of *Sphaeropsis malorum*, as evidenced by the formation of tryptophane from the peptone of Dunham's solution, but no further studies of the proteases of the genus Sphaeropsis appear to have been made, although Reed (18) has recorded the presence of an amidase able to form ammonia from alanin and asparagin in *Glomerella rufomaculans*.

Many of the statements made in the preceding discussion are rendered conclusive by inspection of the data for phosphorus. In the normal fruit the distribution of phosphorus between the three fractions is as follows: Lipoid fraction 30.25 per cent, water-soluble portion of alcohol-ether-water extract 57.22 per cent, and the insoluble portion 12.53 per cent. In the half-decayed fruits there is a very marked decrease in the insoluble fraction from 12.53 to 6.12 per cent; a less marked decrease from 30.25 per cent to 27.40 per cent in the lipoid fraction, and a concurrent gain in water-soluble phosphorus from 57.22 to 66.48 per cent of the total. These figures show that in the earlier stages of the attack the changes which involve phosphorus are predominantly katabolic in nature, and that they affect both lipoid and protein phosphorus, reducing both to simpler water-soluble forms. With the further progress of the disease, there is a further reduction of lipoid phosphorus to 25 per cent,

a decrease of water-soluble phosphorus to 57.89 per cent, which is practically the proportion found in normal tissues, and an increase in fraction 3 to 17.11 per cent, as compared with 12.53 per cent in sound fruits. Here constructive processes are predominant; a portion of the lipoid phosphorus, together with that derived from destruction of the host proteins, has been utilized in the construction of new and complex material, presumably protein in nature, by the blackrot organism. We therefore possess, in the analytical data for nitrogen and phosphorus, an index to the character and amount of the changes in nitrogenous constituents of the fruit brought about during decay. While no attempt to do so has been made in this case, it would appear that a quantitative estimation of the several phosphorus-containing groups and a determination of the relative amounts of proteoses, peptones, and amino acids present at various stages of the progress of the disease would contribute much to our knowledge not only of the changes wrought in the host proteins by the attacking fungus, but also of the extent to which these materials are wholly destroyed or utilized in constructive processes by the parasite. Such determinations will be attempted by the senior author, who contemplates continuing the work here reported.

The most obvious changes in carbohydrate content are those occurring in fraction 2, which contains reducing sugars, disaccharids, trisaccharids, and glucosids, and in fraction 3, which contains the insoluble carbohydrates, as starch, cellulose, and cellulose derivatives. The reducing sugars in fraction 2 are reduced from 0.574 gm. in normal fruit to 0.1619 gm. in half-decayed fruit, and to 0.0609 gm. in wholly decayed tissue; in other words, there is a loss of 89.4 per cent of reducing sugars with the progress of the disease. Disaccharids, which total 4.960 gm. in normal apples, are reduced to 3.651 gm. in half-decayed fruit and to 2.234 gm. in wholly decayed fruits, a loss of 56.94 per cent. The data obtained from the determinations of acidity will be discussed later. It may be said here, however, that the acid content of the sound fruit, which equaled 0.9288 per cent, calculated as malic, was reduced to 0.3563 per cent in the wholly decayed fruit. The steady decrease in sugar content is accompanied by an even larger proportional decrease in acid content, since 71.64 per cent of the acid of sound fruits had disappeared concurrently with a loss of 89.4 per cent of the reducing sugars and 56.94 per cent of disaccharids.

There is also a reduction in the amount of lipoid sugar in fraction 1 as decay proceeds. The first determinations of sugar in this fraction were vitiated by the fact that sufficient precautions were not taken to remove the last traces of chloroform. When the senior author attempted to repeat the determinations upon portions of the lipoid fraction which had been reserved for the purpose, he encountered further difficulties. When a 24-hour hydrolysis with 4 per cent of hydrochloric acid is carried

out upon these fractions, a great variety of products are present in the aqueous solution. Glycerol derived from the hydrolysis of lipoid materials was present in small amounts; phloroglucinol and catechol or some very closely related substance were present in sufficient quantities to give all the usual tests (16), and the presence of other unknown compounds capable of reducing Fehling's solution was not precluded. Precipitation with normal lead acetate and the subsequent removal of the excess of lead with saturated sodium-sulphate solution removed tannin derivatives, but it is impossible to remove glycerol by any method of treatment which does not also cause some loss of sugars. The figures obtained therefore have a comparative rather than an absolute value; they indicate a reduction from 49.97 mgm., the quantity found in normal fruit, to 12.69 mgm. in totally decayed apples, a reduction of 74.61 per cent.

The results of the determination of polysaccharids were distinctly surprising and effectually destroyed any preconceived ideas as to the utilization of these compounds in the metabolism of the fungus. The starch content of the normal apples was 0.321 gm., that of half-decayed fruits 0.344 gm., and that of wholly decayed fruits 0.318 gm. It was consequently evident that conversion of starch into simpler forms had not occurred under the conditions of the experiments. Since Reed (18) demonstrated the presence of a diastase capable of acting vigorously upon both corn and arrowroot starch in enzym powders prepared by alcoholic extraction of apples completely rotted by *Sphaeropsis malorum*, while Thatcher (25) found no diastase in ripe sound apples, this point was subjected to further investigation. Microscopic examination of starch grains from completely decayed apples showed no discoverable erosion of the starch grains. Since this result might be due to inhibition of the secretion of diastase by the fungus in the presence of sugars, as Katz (8) found to be the case with several fungi, the senior author tested the diastatic activity of fresh extracts of pure cultures of *Sphaeropsis malorum* grown upon a variety of media poor or lacking in sugar. In no case was there the slightest activity either upon corn or apple starch. It would therefore appear that the amylase found by Reed was derived from the apple and not from the attacking fungus, and that the disagreement between the results of Reed and of Thatcher can be explained only by the assumption that they were dealing with fruits of differing degrees of maturity. Hawkins (5) found that *Fusarium oxysporum*, *F. radicicola*, and *F. coeruleum* were unable to attack the starch of potato tubers, showing no action upon it even after one week, although extracts of these fungi rapidly digested soluble starch. The same investigator has shown (4) that in the case of the peach, rotting induced by *Sclerotinia cinerea* (Bon) Schröter produces only very slight decrease in the amount of alcohol-insoluble material capable of reducing

Fehling's solution after hydrolysis with dilute hydrochloric acid. About one-third of this material appears to be starch, as determined by digestion with malt diastase.

There was a large and consistent increase in the yield of reducing sugar given by the insoluble residue after five hours' hydrolysis with 2.5 per cent of hydrochloric acid under a reflux condenser. In the normal tissue, the amount of hydrolyzable carbohydrate material, after deducting the amount of starch found by separate determinations, was 0.315 gm. In half-decayed tissue, the quantity had risen to 0.771 gm., and in wholly decayed tissue there was a further increase to 1.086 gm., or a total gain of 0.771 gm. That this hydrolyzable material is not derived from partial decomposition of cellulose or other structural materials is conclusively shown by the fact that after deducting from the total weight of the insoluble residue of normal fruits, 2.100 gm., the weight of the hydrolyzable portion, 0.636 gm. plus the weight of the protein therein contained as indicated by the nitrogen content, 0.2456 gm., we have remaining 1.2184 gm. as the weight of the nonnitrogenous, nonhydrolyzable residue. In the half-decayed fruit, after making similar deductions, there remain 1.0662 gm. as the weight of this residue, while in the wholly decayed fruit this residue amounts to 1.7435 gm. These results were seriously questioned when first obtained; but repeated determinations yielded results which were entirely concordant, leaving no question that there occurs in the course of the disease a very marked increase in both the hydrolyzable and the nonhydrolyzable residues of fraction 3. In the early stages of the disease the nonhydrolyzable portion decreases very considerably, but subsequently increases to 143.08 per cent of its original amount. The increase in hydrolyzable constituents aside from starch is 244.76 per cent. These very large increases can be explained only as being the results of constructive processes carried on by the fungus, in which connection the increase of nitrogen and phosphorus in this fraction is significant.[1] In the face of our almost total absence of knowledge as to the amount of the carbohydrate synthesis occurring in any parasitic fungus and particularly as to the nature of the materials loosely designated as "glycogen," speculation as to the meaning of these results would be fruitless, but they indicate an interesting field of investigation. It is hoped that the study may be continued to the end that some information in regard to the nature of the compounds formed in these synthetic processes may be gained.

The variations in total ash content shown by the analyses are accounted for by the fact that determinations are made upon small aliquot

[1] Hawkins (5) found that there was rather vigorous construction of both hydrolyzable and nonhydrolyzable material by *Fusarium oxysporum* growing on potatoes; the crude fiber content of rotted quarters of tubers was uniformly considerably greater than that of sound quarters used as checks, as was the content of material reducing Fehling's solution after acid hydrolysis. The relative amounts of such materials produced by *F. oxysporum* are very much smaller than those found by us for *Sphaeropsis malorum*.

parts of each fraction and not upon large single samples. The presence of waxy and resinous bodies in the lipoid fraction also contributes to the difficulty met in securing accordant results. In normal fruits 68.22 per cent of the total ash is present in fractions 2, with 31.11 per cent in fraction 3. In half-decayed material these percentages become 78.66 and 21.81, respectively, while when decay becomes complete 85.11 per cent is present in fraction 2 and only 14.89 per cent in fraction 3. There is clearly a steady transfer of mineral elements from insoluble combinations with constituents of fraction 3 into less complex, readily soluble forms as the disease proceeds. At the same time there is a reduction in the originally very small quantity of ash in the lipoid fraction practically to zero, the amounts found in this fraction for wholly decayed fruits being uniformly too small to weigh.

As originally planned, the present study also contemplated the determination of the amounts of tannins and tannin derivatives present in normal and diseased fruits, to the end that some information as to the effect of the growth of the fungus upon these compounds might be secured. A review of the literature resulted in the bringing together of a number of methods which were tried out in a comparative way, in part by the authors upon apples and pears, in part by Dr. F. A. Wolf in the course of his work in the same laboratory upon other plant material (28). The results were of such discordant character as to be entirely valueless. It is clear that we have as yet no methods of estimating tannins which are sufficiently quantitative to be dependable when a fruit very low in tannin content is employed and when the purpose in view is the recognition of small alterations in this content.

CHANGES PRODUCED BY SPHAEROPSIS MALORUM IN APPLES IN ARTIFICIAL CULTURE

In order that the analytical results reported in the preceding pages might be compared with results obtained when pure cultures of *Sphaeropsis malorum* were allowed to act upon mature sterile apple tissue under the most favorable conditions for growth obtainable in the laboratory, a number of experiments with such cultures were made.

On June 21 a carefully selected lot of sound, mature apples were ground in the manner previously described, 100-gm. samples were weighed into Erlenmeyer flasks, plugged with cotton, and sterilized in an autoclave for three successive days. Half the flasks were then inoculated from a pure culture of *Sphaeropsis malorum;* the remaining flasks were kept as sterile checks. All were then incubated until August 15 at 32° C. Dry-weight determinations upon a 100-gm. portion of the original material gave a weight of 12.600 gm. At the end of 54 days of incubation, sterile and inoculated flasks were opened and the contents of each separated into the three fractions. The dry weight of each fraction was then determined. The results are given in Table III.

TABLE III.—*Dry weights (in grams) of fractions 1, 2, and 3 in sterile checks and in artificial cultures of Sphaeropsis malorum after 54 days' incubation*

Item.	Fraction 1.	Fraction 2.	Fraction 3.	Total.
Sterile check	0.850	9.270	2.384	12.404
Inoculated flask	.470	4.170	3.065	7.705
Changes in fractions in the inoculated flasks	− .380	− 5.100	+ .681	− 4.699
Percentage of gain or loss in inoculated flasks	−44.7	−55.02	+28.55	−37.97

It is unnecessary to point out that the conditions in an artificial inoculation upon finely ground pulp which has been sterilized under pressure differ fundamentally from those surrounding an infection of the whole fruit under natural conditions. Sterilization brings about a very considerable hydrolysis of disaccharids and of more complex carbohydrate materials, so that a much larger supply of reducing sugars is available to the fungus. The finely ground character of the material permits the rapid penetration of the material by the fungus, and there is consequently an exceedingly rapid growth. The enzyms of the fruit are destroyed, but there is opportunity for rapid spontaneous oxidations as a result of the free access of air through the cotton plugs, as is evidenced by a loss of 0.196 gm., or 1.55 per cent, of the dry weight of the check during the period of incubation. The inoculated flask meanwhile lost 4.699 gm., or 37.97 per cent, a loss almost 25 times as great as that occurring in the sterile control.

In artificial inoculations there is a considerable increase in the amount of alcohol-ether-water-insoluble material constituting fraction 3. In the sample for which data are given in Table III, the dry weight of this fraction was 3.065 gm., as compared with 2,384 gm. in the normal tissue, a gain of 0.681 gm., or 28.55 per cent. These results afford further evidence of the fact brought out by the detailed analyses of naturally infected fruits, namely, that there is a very considerable conversion of soluble to insoluble material as a result of the activities of the fungus. This conversion takes place with greater rapidity in comparison with the loss of soluble constituents from fraction 2 in unsterilized fruits, since in the fruit entirely decayed as a result of natural infection a loss of 2.659 gm. from fraction 2 was accompanied by a gain of 1.345 gm. in fraction 3, while in the artificial inoculations, a loss of 5.100 gm. from fraction 2 was accompanied by a gain of only 0.681 gm. in fraction 3. These very considerable differences indicate that there are large differences between the metabolic activities of the fungus when grown upon sterilized, aerated pulp and those occurring in natural infections. They emphasize the obvious fact that very great possibilities of error exist wherever one attempts to reason back from the results obtained with artificial cultures and to construct therefrom a picture of the life processes of a pathogenic organism pursuing its life cycle under natural conditions.

Comparisons of the carbohydrate content of artificial cultures with sterile checks were made upon material collected June 27, when the fruits had become fully ripe and somewhat soft. Four identical 100-gm. samples were prepared from one lot of pulp, sterilized for three successive days, and two were then inoculated while two were kept as sterile checks. All were incubated for 42 days. One each of the inoculated and the sterile flasks were then opened and the contents extracted for 12 hours with 95 per cent alcohol, the other pair being extracted for the same period with water. After the removal of the noncarbohydrate material from the extracts, determinations of the reducing and nonreducing sugars were made by the methods previously described. The results are summarized in Table IV.

TABLE IV.—*Carbohydrate content (in grams) of water and alcohol extracts of sterile checks and cultures of Sphaeropsis malorum upon apple pulp after 42 days' incubation, 100 gm. of finely ground pulp in each flask*

Extract.	Reducing sugars.	Nonreducing sugars.	Total sugar after hydrolysis.
Water extract:			
Sterile check	1.736	0.328	2.064
Inoculated	.960	.1336	1.0936
Alcohol extract:			
Sterile check	7.496	.1427	7.638
Inoculated	2.128	.068	2.196

Alcohol extraction recovered from the sterile check a total of 60.62 per cent of the original dry weight as sugars, and from the inoculated material only 17.42 per cent, a difference of 43.20 per cent, or 5.442 gm. in an original dry weight of 12.600 gm. That monosaccharids are rapidly attacked by the fungus while disaccharids are much more slowly reduced is indicated by the data of Table VI as well as by that presented in Table II.

Since *Sphaeropsis malorum* obtains the energy necessary for its growth mainly from the oxidation of carbohydrates, an attempt was made to secure information as to the nature of the products resulting from carbohydrate decomposition. Determinations of the acid and alcohol content of fruits in various stages of decay from blackrot, with check determinations upon sound fruits, were carried out.

ACIDITY OF NORMAL AND DISEASED APPLES

Sound, partially decayed, and wholly decayed apples were selected and material was prepared and ground exactly as in the preparation of other samples. Duplicate 40-gm. portions of each lot were weighed off, each portion was placed in a 500 c. c. flask, and 200 c. c. of distilled water added thereto. One set of flasks was then placed on a water bath and kept at 100° C. for four hours, the duplicate set meanwhile being kept at room temperature. All were thoroughly shaken at short intervals.

At the end of four hours the contents of the flasks were poured upon large filters, allowed to drain, and washed with distilled water until the total volume of the filtrate and washings equalled 500 c. c. A convenient portion of each filtrate was then titrated with *N/20* sodium hydroxid. As the tissues of the apple contain a natural indicator which develops a marked color upon neutralization, the method of titration employed was that described by Schley (23). The results given in Table V state in each case the number of cubic centimeters of *N/20* sodium hydroxid required to neutralize the acids in the clear filtrate from 40 gm. of fresh pulp.

TABLE V.—*Acidity in normal and diseased tissue of apple expressed in cubic centimeters of N/20 sodium hydroxid required to neutralize the clear filtrate from 40 gm. of fresh pulp after four hours' extraction with water*

Extraction and date.	Normal.	Half-decayed.	Wholly decayed.
Average for samples taken June 17:			
Cold water	8.5	4.3	3.4
Hot water	11.7	4.6	4.5
Average for samples taken June 22:			
Cold water	9.7	6.0	3.3
Hot water	9.7	7.5	4.0

It is, of course, recognized that this method gives only comparative results, that a considerable portion of the acid content is not extracted by water, and that much higher figures would have been obtained had the titration been made directly upon the pulp suspended in water. It may be pointed out, however, that when titrations are made by the last-mentioned method, the diffusion of acids out of the tissues continues for many hours and at slower rates in diseased than in normal fruits, as experiments in this laboratory have shown. Hence, the employment of a method which combines rapidity of performance with the attainment of consistent approximate results. As a check upon determinations made upon samples from the field, artificial inoculations and sterile checks containing 40 gm. each of pulp were prepared, sterilized, and inoculated for 33 days in all respects as previously described, and 150 c. c. of distilled water were then added to each flask. The flasks were heated on the water bath for 30 minutes, and duplicate portions were taken for titration. The neutralization of the acids of the sterile check required 66 c. c. of *N/20* sodium hydroxid, for the inoculated pulp 3 c. c. were required. The acidity of the sterile check was therefore 22 times that of the culture. In natural infections, there is a reduction of acidity to about one-third that found in sound fruits. The growth of *Sphaeropsis malorum* upon the apple, whether under natural or artificial conditions, is therefore accompanied by a considerable reduction in the acidity of the tissues. Reed (18), in the course of his studies of the enzymic activities of *Glomerella rufomaculans*, observed that this fungus produced an alkaline reaction in originally

acid synthetic media, and also that the acidity of juice from infected apples was materially decreased. Further studies of this point by Reed and Grissom (20) brought out the fact that this production of alkalinity in culture media is due, at least in part, to the production of carbon dioxid and the resulting formation of carbonates, in part to the formation of ammonia from the peptone of the media, and in part to the formation of ammonia and purin and hexone bases as a consequence of the autolysis of the proteins of the fungus (19). That none of these causes is active in the reduction of acidity produced by *Sphaeropsis malorum* is evident from the fact that the nitrogen of the alcohol-ether-water-soluble fraction, as well as the ammonia therein present, steadily decreases as the disease proceeds, a fact quite precluding the possibility of the formation of hexone or purine bases. The results point rather conclusively to the destruction of organic acids by oxidation as a cause of the observed facts. That this is the true explanation is borne out by the fact that Wolf, in the course of studies conducted in this laboratory (28), has observed that a species of Phoma is capable of decreasing the acidity of several species of Citrus without a concurrent increase in the soluble nitrogenous constituents. Cooley (3) and Hawkins (4), in their studies of the chemical changes produced in the peach by *Sclerotinia cinerea*, found that the development of the fungus was accompanied by a marked rise in the acidity of the tissues, and Cooley showed that this was due to the production of oxalic acid, which was absent from the plum and peach juices used as culture media. That we are here dealing with activities of an entirely different character is evident; there is absence of acid formation with progressive decomposition of the acids naturally present in the fruit.

ALCOHOL DETERMINATIONS IN SOUND AND DISEASED APPLES

Samples for alcohol determination were prepared from sound, half-decayed, and wholly decayed fruits by grinding, weighing 200-gm. samples into distillation flasks, and adding 500 c. c. of distilled water to each sample. The flasks were then attached to condensers and distillation continued until alcohol had ceased to come over and a total of 100 c. c. of distillate had been obtained from each flask. Determinations of alcohol in these distillates were made according to the method originally devised by Nicloux (14), as described by Pringsheim (17), which is based upon the oxidation of the alcohol by potassium bichromate. Five c. c. of each distillate was placed in a beaker, a small measured quantity of *N/20* potassium bichromate added, 35 c. c. of concentrated sulphuric acid poured in, and the solution heated. The solution was then continuously stirred while *N/20* potassium bichromate was cautiously added from a burette until the completion of the oxidation was indicated by a change in the color of the solution from greenish blue to yellowish green. The comparative results are as follows: For normal apples, 5 c. c. distillate required 19.4 c. c. of potassium bichromate; for half-decayed fruits, 5 c. c. required 22.3 c. c. of potassium bichromate; for completely decayed fruits, 5 c. c. required 30.6 c. c. of potassium bichromate.

A further comparison of the alcohol content of sterile tissues and the artificially inoculated material was made. Forty-gm. samples of pulp prepared and sterilized as usual and incubated for 30 days after inoculation of half the number of flasks were employed. Two hundred c. c. of water were added to each flask and distillation continued until alcohol ceased to come over and 50 c. c. of distillate had been collected, when 5 c. c. samples were titrated. These distillates are two-fifths as strong as those obtained from the fresh material, when amounts of pulp and distillate are compared. When corrections are made for this difference, the results for the check and the inoculated material are for 5 c. c. of distillate, 7.5 and 10.5 c. c., respectively.

The experiments agree in showing that there is a considerable increase in the alcohol content of tissues invaded by the blackrot fungus, whether under natural or artificial conditions; moreover, they show that this increase proceeds at an equal pace with the disappearance of soluble carbohydrates from the affected fruits.

SUMMARY

A quantitative analytical comparison of the chemical composition of normal mature Red Astrachan apples with that of similar fruits in two stages of decay as a result of attack by *Sphaeropsis malorum* reveals certain well-marked alterations in composition which proceed at an equal pace with the progress of the disease.

(1) The loss of water from the affected tissues is small, amounting to 4.61 per cent of the original weight in the case of fruits which had just reached the stage of complete decay.

(2) There is a very considerable reduction in the total solids present, amounting to 6.80 per cent in totally decayed fruits.

(3) There is a very marked reduction, concurrently with the progress of the disease, in the amount of the constituents removed by successive extractions of the pulp with alcohol, ether, water, and alcohol. In sound fruit these make up 78.94 per cent of the total solids, in wholly decayed fruits 65.14 per cent. This reduction goes on very slowly in the earlier stages of the disease but quite rapidly in the later stages.

(4) There is a decrease, followed by an increase, in absolute as well as in relative amount of lipoid constituents extracted by alcohol or ether, and precipitated from watery emulsion by chloroform, with the progress of the disease. In half-decayed material these constituents are reduced to 83.17 per cent of the absolute amount found in sound fruits; in wholly decayed material they are increased to 121.48 per cent of the total content of the sound apples. In the onset of the disease there is active attack and transformation of the lipoid constituents of the host; later there is rapid construction of lipoid materials by the parasite.

(5) The nitrogen extracted by alcohol, water, and ether, which represents the nitrogen of proteoses, peptones, polypeptids, amino acids, and nitrogen bases, steadily decreases with the progress of the disease, as

does the ammonia extracted by these solvents. There is consequently no reduction of acidity through the formation of purin and hexone bases, as was found to be the case in *Glomerella rufomaculans*.

(6) The nitrogen of the alcohol-ether-water-insoluble fraction, representing protein nitrogen, increases steadily with the progress of the disease. This increase runs nearly parallel with the decrease in nonprotein nitrogen in the soluble fraction, indicating that the parasite utilizes these forms of nitrogen in the synthesis of proteins.

(7) There is a steady but small decrease in the total nitrogen present, due to complete decomposition of some of the nitrogenous constituents with the escape of ammonia.

(8) The phosphorus of both lipoid and insoluble fractions is materially decreased in the half-decayed fruits, with a concurrent increase in the amount of phosphorus extracted by alcohol, water, and ether. In completely decayed fruit there is a further reduction in lipoid phosphorus, a marked increase in soluble phosphorus, and a very large increase in insoluble or protein phosphorus. The organism is able to reduce both the lipoid and the protein phosphorus of the host tissues to simpler water-soluble forms and to utilize their phosphorus in the construction of new and complex phosphorus-containing materials.

(9) There is a steady transfer of mineral elements from the insoluble to the soluble fraction, the percentage of the total ash present in fraction 2 increasing from 68.22 to 85.11 per cent in the course of decay.

(10) There is a rapid decrease in the content of reducing sugars, disaccharids, and lipoid sugars as the disease proceeds. Of these classes of carbohydrates, disaccharids are least completely utilized, totally decayed fruits having 45.04 per cent of the disaccharid content, but only 10.6 per cent of the monosaccharid content, of normal fruits.

(11) Starch is not attacked by the fungus, its amount remaining unchanged throughout the progress of decay.

(12) There is a rapid increase in the hydrolyzable carbohydrate material other than starch, present in fraction 3. In the early stages of decay there is a slight decrease in the nonhydrolyzable portion of this fraction, which is followed by a large increase. Invasion by species of Sphaeropsis is characterized by a large increase in materials convertible into reducing sugars by hydrolysis for five hours with 2.5 per cent hydrochloric acid and by a less rapid increase in substances not affected by such hydrolysis. The natures of the compounds thus synthesized has not been determined.

(13) There is a progressive decrease in the acid content of the fruits from 0.9288 per cent for sound apples to 0.3086 per cent in those which become completely decayed. In artificial inoculations upon sterile apple pulp the reduction of the acid content is more rapid and more complete.

(14) The progress of the disease, both in natural infections and in artificial inoculations upon sterile apple pulp, is accompanied by a large increase in the alcohol content of the tissues.

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FORMATION OF HEMATOPORPHYRIN IN OX MUSCLE DURING AUTOLYSIS

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INTRODUCTION

In the course of a series of autolysis experiments with ox muscle certain changes in the color of the tissue were noted, which, on closer study, were found to be due to the formation of hematoporphyrin. While this compound is a well-known derivative of hemoglobin, yet this is the first instance, so far as the author has been able to determine by a careful search in the literature, where the formation of hematoporphyrin in this manner has been reported. Since the method and place of the production of hematoporphyrin in the human body are not at all clearly understood, the results of these observations are offered with the hope that they may throw some light on that subject.

EXPERIMENTAL WORK

A series of experiments was conducted for the purpose of studying the chemical changes which take place in ox muscle during autolysis. A detailed description of these experiments has been reported in a previous paper by Hoagland and McBryde,[2] and only such details as seem pertinent to this paper will be described here.

Both aseptic and antiseptic experiments were conducted, but the observations now to be reported pertain to the aseptic experiments. These were carried on under strict bacteriological control. Thirty-six samples of muscular tissue from the hind quarter of an ox were secured by aseptic methods and were transferred to sterile covered crystallizing dishes. The dishes were sealed by means of adhesive tape and were incubated at 37° C. for periods ranging from 7 to 220 days. The samples weighed from 274 to 512 gm., the average being 377 gm. Out of the 33 samples 21 showed visible evidences of bacterial contamination and were discarded. Of the remaining 12 samples 9 were submitted to careful bacteriological examination and proved to be sterile. The remaining 3 samples, which were incubated for 103, 123, and 220 days, respectively, showed no apparent evidences of bacterial growth, and while they were not submitted to careful bacteriological examination, yet their appearance

[1] The author desires to extend his thanks to Dr. C. N. McBryde, of the Biochemic Division, who prepared the samples of muscle for incubation and who exercised bacteriological control over the experiments.

[2] Hoagland, Ralph, and McBryde, C. N. Effect of autolysis upon muscle creatin. *In* Jour. Agr. Research, v. 6, no. 14, p. 535–547. 1916. Literature cited, p. 546–547.

as compared with that of the 9 other samples was reasonably good evidence that they also were sterile.

Early in the course of the experiments it was noted that the exposed surfaces of the samples had turned light brown in color, in contrast to the red color of fresh lean beef, while the surface that rested on the bottom of the dish had become bright pink in color. When a cross section was cut through a sample, it was found that the brown color extended to a depth of about one-fourth of an inch, the width of the zone increasing somewhat with the period of incubation, while the interior of the sample was of a uniform bright pink color. In a few cases the meat samples rested in the dishes in such a way as to pocket some of the exuded juice and protect it from the air. In these instances the juice had a peculiar dark purplish red color, as compared with the muddy brown color of the juice that had been exposed to the air. After the meat was ground for analysis it soon turned brown in color. At first it was assumed that the production of the pink to purplish red color was due to a simple reduction of oxyhemoglobin to hemochromogen. A spectroscopic examination of the 0.9 per cent sodium-chlorid extract of the sample that had been incubated for 7 days showed that the coloring matter was not hemochromogen, and that oxyhemoglobin and hemoglobin also were absent. These facts led to a careful study of the color of a number of samples incubated for various periods of time, with the following results:

SAMPLE INCUBATED FOR 7 DAYS.—The exposed surface of the sample was light brown in color, while the surface that rested on the bottom of the dish was bright pink. When a cross section was cut through the sample, the brown color was found to extend to a depth of about one-fourth of an inch from the surface, while the interior of the sample was uniformly bright pink in color. The 0.9 per cent sodium-chlorid extract of the meat had a light-pink color. On spectroscopic examination the extract showed a fairly distinct narrow band at the left of, and extending just over, the D line, and a wider and less distinct band midway between the D and the E lines. On the addition of hydrazin hydrate the bands at first became more distinct, but after a time disappeared. The fact that the absorption bands were not readily affected by hydrazin hydrate and that no absorption bands of either hemoglobin or hemochromogen appeared after the addition of this reagent indicates that the color of the solution was due neither to oxyhemoglobin nor to hematin. The spectrum of the solution was, of course, neither that of hemoglobin nor that of hemochromogen.

SAMPLE INCUBATED FOR 103 DAYS.—This sample of meat showed the brown outer zone and the pink interior common to all of the incubated samples. The meat rested in the dish in such a way as to pocket a considerable quantity of meat juice, perhaps 20 c. c., out of contact with the air. This juice had a peculiar dark purplish red color and showed the

following spectrum: A heavy narrow band, with sharply defined edges, immediately at the left of the D line; and a second band, two to three times as wide as the first band, but not quite so heavy, and with less sharply defined edges, midway between the D and the E lines. The addition of hydrazin hydrate did not affect the color or spectrum of the solution.

SAMPLE INCUBATED FOR 123 DAYS.—This sample had the usual brown outer zone and pink interior. When ground and extracted with water, the pink inner portion yielded a light straw-colored extract tinged with pink. The following spectrum was observed: A fairly heavy band immediately at the left of the D line; and a lighter and wider band between the D and the E lines.

The extract was tested for bile pigments by means of Hammarsten's test, with negative results.

SAMPLE INCUBATED FOR 220 DAYS.—The brown outer zone had extended to a depth of nearly an inch, leaving only a small inner portion that was pink. The pink-colored portion when ground and extracted with water yielded a light straw-colored extract that showed the following spectrum: A heavy narrow band just at the left of the D line; and a wider, heavy band midway between the D and the E lines. The addition of hydrazin hydrate did not affect the color or the spectrum of the solution.

A summary of the observations which have been made concerning the effect of autolysis upon the natural red color of ox muscle leads to the conclusion that the pink or purplish red color which was developed in the interior of the samples of muscular tissue and in the exuded juice that had been protected from the air was due to hematoporphyrin that had been formed by the reduction of oxyhemoglobin. This conclusion is supported by the following evidence: (1) The spectrum of the color and its behavior toward reducing agents correspond with those of hematoporphyrin; (2) the color was formed only in the absence of oxygen, a condition necessary for the formation of hematoporphyrin, and it was destroyed on exposure to the air; (3) conditions under which hematoporphyrin is formed in the body indicate that this compound probably results from the action of certain intracellular enzyms upon free hemoglobin, and it is reasonable to expect that a similar change might occur in the coloring matter of muscular tissue during autolysis, provided that suitable conditions are maintained; (4) the pink to purplish red color developed in the tissue and exuded juice during autolysis is characteristic of hematoporphyrin. In all essential properties, the substance which imparted the pink to purplish red color to the interior of the incubated meat samples, and to the exuded meat juice that was protected from the air, correspond to hematoporphyrin.

SIGNIFICANCE OF THE FORMATION OF HEMATOPORPHYRIN DURING THE AUTOLYSIS OF MUSCULAR TISSUE

It seems proper to call attention to the possible significance of the formation of hematoporphyrin under the conditions which have been described not only as regards the excretion of this compound by the body under pathological conditions but also as related to the normal transformation of hemoglobin into bile pigments.

OCCURRENCE.—Hematoporphyrin occurs in traces as a constituent of the normal urine of man and of the higher animals, and it has been found in the feces. It occurs most frequently, and in largest quantities, as a constituent of the urine under pathological conditions, particularly in cases of poisoning with sulphonal, trional, tetronal, lead, and phosphorus; in case of fevers and of gastric and intestinal hemorrhages; in diseases of the liver; and in various cases of acute infectious diseases—for example, tuberculosis, nephritis, pleuritis, rheumatism, and Addison's disease—and under certain other conditions.

FORMATION IN THE BODY.—Comparatively little appears to be known as to how or where hematoporphyrin is formed in the body.

Oswald [1] states that very little is known concerning the place or method of production of hematoporphyrin in the body. He discusses the hypotheses proposed by various investigators concerning the subject and concludes that the evidence seems to indicate that hematoporphyrin originates in the blood stream, particularly since this pigment is often found in the urine in cases of hemoglobinuria. In concluding the discussion on the subject the author states:

> Jedenfalls ist die Frage nach dem Orte der Hämatoporphyrinbildung noch nicht entschieden. Sie bedarf noch weiterer Bearbeitung.

Although a diligent search has been made in the literature relating to hematoporphyrin, no satisfactory explanation, based upon experimental evidence, has been found as to the method of formation or the seat of production of that compound in the body.

The experiments which are reported in this paper show that the striated muscular tissue of the ox contains enzyms which, under anaerobic conditions, readily reduce oxyhemoglobin to hematoporphyrin. These findings appear to offer a satisfactory explanation as to the method and source of production of hematoporphyrin in the body.

As has been previously noted, hematoporphyrin may occur in the urine in very small quantities under physiological conditions; but it occurs most often and in largest quantities under certain pathological conditions, notably (1) those obtaining in cases of poisoning or disease where the liver cells are destroyed or inactivated and (2) those obtaining in case of certain diseases or other conditions that cause an abnormal liberation of free hemoglobin into the blood stream.

[1] Oswald, Adolph. Lehrbuch der chemischen Pathologie. S. 171–176. Leipzig, 1907.

The generally accepted view concerning the disposal of the free hemoglobin in the blood stream under normal conditions is that the hemoglobin is converted directly into bile pigments by the liver. Such being the case, it would seem that when an abnormal quantity of hemoglobin is liberated into the blood stream, or when the activity of the liver is greatly decreased by disease or other causes, that hemoglobin rather than hematoporphyrin would be excreted in the urine.

The results of the experiments reported in this paper, which show that the striated muscular tissue of the ox has the property of readily and completely reducing oxyhemoglobin to hematoporphyrin, not only indicate a source and method of production of this compound in the body but likewise suggest that hematoporphyrin may be an intermediate product in the transformation of hemoglobin into bile pigments. The presence of hematoporphyrin-producing enzyms in muscular tissue can hardly be regarded as without physiological significance.

In this connection a statement by Matthews [1] is significant. He asserts that it is one of the functions of the liver to pick out the hematoporphyrin from the blood, where it occurs normally in small quantities, and convert it into a harmless bile pigment.

SUMMARY

The results of the experiments reported in this paper may be summarized as follows:

(1) The striated muscular tissue of the ox contains enzyms which, under anaerobic conditions, readily and completely reduce oxyhemoglobin to hematoporphyrin.

(2) It appears very probable that hematoporphyrin may be a regular intermediate product in the transformation of hemoglobin into bile pigments.

[1] Matthews, A. P. Physiological Chemistry. p. 422. New York, 1915.